MONIQUE COLOMBO

BENEFICIAL PROPERTIES AND SAFETY OF LACTIC ACID BACTERIA ISOLATED FROM THE DAIRY PRODUCTION ENVIRONMENT

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 30 de junho de 2017.

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I thank God for giving me a better life than I could ever ask for.

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SUMMARY

LIST OF TABLES v

LIST OF FIGURES vi

RESUMO vii

ABSTRACT ix

INTRODUCTION 1

OBJECTIVES 2

CHAPTER 1 - A BRAZILIAN VIEW OF THE POTENTIAL USE OF PROBIOTIC/BENEFICIAL BACTERIA BY THE DAIRY INDUSTRY 3

ABSTRACT 5
INTRODUCTION 6
HEALTHY LIFESTYLE AND GOOD NUTRITION 6
DEFINITION OF PROBIOTIC MICROORGANISMS 7
BENEFICIAL EFFECTS OF PROBIOTIC MICROORGANISMS 9
PROBIOTIC SELECTION CRITERIA 13
DAIRY PRODUCTS AS VEHICLES FOR PROBIOTIC CULTURES 15
FUTURE PROSPECTS: EXPANSION OF THE DAIRY INDUSTRY THROUGH SCIENTIFIC RESEARCH 17
CONCLUDING REMARKS 19
REFERENCES 19

CHAPTER 2 - BENEFICIAL PROPERTIES OF LACTIC ACID BACTERIA NATURALLY PRESENT IN DAIRY PRODUCTION SYSTEMS 25

ABSTRACT 27
MATERIALS AND METHODS 28
Screening 28
Samples and microbiological analysis 28
Lactic acid bacteria isolation and characterisation 28
Gastric pH resistance 29
Bile resistance 29
Molecular identification 30
Detection of enzymatic activity 30
Beneficial properties 32
Resistance to simulated gastric and intestinal conditions 32
Aggregation and co-aggregation properties 32
Bile salt deconjugation 34
β-galactosidase activity 34
Lactose assimilation 34
RESULTS AND DISCUSSION 34
Screening 34
Beneficial properties 41
CONCLUSION 45

ACKNOWLEDGEMENTS 46
REFERENCES 46

CHAPTER 3 - VIRULENCE POTENTIAL AND ANTIMICROBIAL RESISTANCE OF BENEFICIAL LACTIC ACID BACTERIA ISOLATED FROM DAIRY PRODUCTION SYSTEMS 50

ABSTRACT 52
1. INTRODUCTION 53
2. MATERIAL AND METHODS 54
2.1. Bacterial strains and growth conditions 54
2.2. Virulence activity 55
2.3. Biogenic amines production 55
2.4. Antibiotic resistance 56
2.5. Detection of virulence and resistance genes 56
3. RESULTS AND DISCUSSION 59
4. CONCLUSION 63
ACKNOWLEDGEMENTS 63
REFERENCES 63

CHAPTER 4 - TECHNOLOGICAL PROPERTIES OF LACTIC ACID BACTERIA OBTAINED FROM A DAIRY PRODUCTION ENVIRONMENT AND DEVELOPMENT OF A FERMENTED MILK WITH THE BENEFICIAL STRAIN LACTOBACILLUS CASEI MRUV6 69

ABSTRACT 70
1. INTRODUCTION 71
2. MATERIALS AND METHODS 72
2.1. Bacterial strains and growth conditions 72
2.3.2. *L. casei* MRUV6 populations monitoring during storage 74
2.3.3. L. casei MRUV6 resistance to bile during storage 74
2.3.4. Expression of bile salt hydrolase (bsh) gene 74
3. RESULTS AND DISCUSSION 76
4. CONCLUSION 82
REFERENCES 82

APPENDIX 90

LIST OF TABLES

CHAPTER 2

Table 1. PCR primers and conditions used for the detection of genes implicated in beneficial properties in lactic acid bacteria strains. 31

Table 2. Enzymatic profile of the studied 15 lactic acid bacteria (LAB) strains determined by APIZYM test. 40

Table 3. Survival of selected 15 lactic acid bacteria (LAB) strains to in vitro gastrointestinal conditions (gastric and intestinal phases). 43

Table 4. Autoaggregation of lactic acid bacteria (LAB) and coaggregation rates between LAB and *L. monocytogenes* Scott A, *E. faecalis* ATCC 19443 and *L. sakei* ATCC 15521 (%), cell hydrophobicity, lactose assimilation, presence of genes associated to beneficial properties tested in 15 selected LAB. 44

CHAPTER 3

Table 1. PCR primers and conditions used for the detection of genes implicated in antibiotic resistance and virulence in LAB strains 57

Table 2. Phenotypic and genotypic antibiotic resistance and resistance and virulence genes detected by PCR of lactic acid bacteria. 62

CHAPTER 4

Table 1. PCR primers 75

Table 2. Technological potential results of 15 LAB strains. 77

Table 3. Mean counts of *L. casei* MRUV6 in fermented milk stored at 4 °C and 10 °C during 35 days in MRS-V agar and MRS-B (results are expressed as $\log_{10}$ CFU/g). 79

APPENDIX

Table 1. Screening for searching of LAB isolates in dairy environment. 90

Table 2. Origin of isolation and isolate media used for selected strains of LAB. 90

Table 3. Enzymatic profile of the studied 16 LAB strains determined by APIZYM test. 91

LIST OF FIGURES

CHAPTER 1

Figure 1. Probiotic action mechanisms. 11

Figure 2. Beneficial effects of probiotic bacteria. 12

CHAPTER 2

Figure 1. Resistance of LAB to effect of low pH as determined at 0h and 3h in non-growing conditions (results are expressed as $\log_{10}$ CFU/mL) and growth of LAB for 18h after been exposed to the effect of low pH for 3h (results are expressed as OD 650 nm determined on microplate reader). A: *Lactobacillus casei* MSI1; B: *Lb. casei* MSI5; C: *Lb. casei* MRUV1; D: *Lb. casei* MRUV6; E: *Lb. acidophilus* MVA3; F: *Lb. nagelli* MSIV4; G: *Lb. harbinensis* MSI3; H: *Lb. harbinensis* MSIV2; I: *Lb. fermentum* SIVGL1; J: *Lb. plantarum* MLE5; K: *Lb. plantarum* MSI2; L: *Pediococcus pentosaceus* MLEV8; M: *P. acidilactici* MSI7; N: *Weissella paramesenteroides* MRUV3; O: *W. paramesenteroides* MSAV5. The white bars represent the counts of the LAB strains at the initial time (zero) and the grey bars represent the count after 3 h incubated in the different pH treatments. The solid line represents the values of optical density in the different pH treatments..... 37

Figure 2. Resistance of LAB to effect of bile salts as determined at 0h and 4h in non-growing conditions (results are expressed as $\log_{10}$ CFU/mL) and growth of LAB for 18h after been exposed to the effect of bile salts for 4h (results are expressed as OD 650 nm determined on microplate reader). A: *Lactobacillus casei* MSI1; B: *Lb. casei* MSI5; C: *Lb. casei* MRUV1; D: *Lb. casei* MRUV6; E: *Lb. acidophilus* MVA3; F: *Lb. nagelli* MSIV4; G: *Lb. harbinensis* MSI3; H: *Lb. harbinensis* MSIV2; I: *Lb. fermentum* SIVGL1; J: *Lb. plantarum* MLE5; K: *Lb. plantarum* MSI2; L: *Pediococcus pentosaceus* MLEV8; M: *P. acidilactici* MSI7; N: *Weissella paramesenteroides* MRUV3; O: *W. paramesenteroides* MSAV5. The white bars represent the counts of the LAB strains at the initial time (zero) and the grey bars represent the count after 4 h incubated in the different bile treatments. The solid line represents the values of optical density in the different bile treatments..... 38

RESUMO

COLOMBO, Monique, D.Sc., Universidade Federal de Viçosa, junho de 2017. **Propriedades benéficas e segurança de bactérias ácido lácticas isoladas de ambiente de produção leiteiro.** Orientador: Luis Augusto Nero. Coorientadores: Svetoslav Dimitrov Todorov e Antônio Fernandes de Carvalho.

Bactérias ácido lácticas (BAL) foram isoladas do ambiente de produção de leite e avaliadas quanto ao potencial benéfico. Testes preliminares e análise por PCR foram aplicados para selecionar e identificar através de sequenciamento de rRNA 16S 15 cepas de BAL: *Lactobacillus* (n = 11; *Lb. casei* MSI1, *Lb. casei* MSI5, *Lb. casei* MRUV1, *Lb. casei* MRUV6, *Lb. acidophilus* MVA3, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSI3, *Lb. harbinensis* MSIV2, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5 e *Lb. plantarum* MSI2), *Pediococcus* (n = 2; *P. pentosaceus* MLEV8 e *P. acidilactici* MSI7) e *Weissella* (n = 2; *W. paramesenteroides* MRUV3 e *W. paramesenteroides* MSAV5). Todas as linhagens selecionadas apresentaram resistência ao baixo pH e à presença de sais biliares. O teste API ZYM foi realizado para caracterizar a atividade enzimática entre as cepas e foi observada elevada atividade β-galactosidase em 13 delas. Todas as cepas apresentaram alta taxa de sobrevivência ao suco gástrico e as condições intestinais simulados, capacidade de auto-agregação e co-agregação com micro-organismos indicadores e alta hidrofobicidade da superfície celular. A maioria das cepas foi positiva para os genes de adesão *map* e *EFTu*. Os resultados de deconjugação de sais biliares mostraram forte desconjugação para todas as cepas. Todas as cepas mostraram bons resultados para assimilar lactose. Após esta etapa de caracterização do potencial benéfico, as 15 BAL foram avaliadas quanto ao potencial de virulência e de resistência antimicrobiana. A produção de fatores de virulência (hemólise, gelatinase, lipase, desoxirribonuclease e aminas biogênicas: lisina, tirosina, histidina e a ornitina) foi avaliada por métodos fenotípicos, a 25 °C e 37 °C, bem como a resistência a 17 antibióticos. Os isolados foram também submetidos à análise de PCR para identificar a presença de 49 genes associados a fatores de virulência. Nenhuma das cepas apresentou atividade hemolítica, produção de gelatinase, lipase, desoxirribonuclease e aminas biogênicas. Das 15 cepas selecionadas, para 12 tipos de antibióticos no método de difusão em disco, todas as amostras foram resistentes à oxacilina e sulfa/trimetoprim, 14 foram resistentes a gentamicina, 11 foram resistentes a clindamicina, nove cepas foram resistentes à vancomicina, oito cepas para rifampicina, cinco foram resistentes a eritromicina, quatro foram resistentes à tetraciclina, duas cepas foram resistentes à ampicilina, uma cepa foi resistente ao cloranfenicol e nenhuma

apresentou resistência ao imipenem. Para um teste quantitativo do antibiograma, 5 antibióticos em fitas Etest® (bioMérieux) foram selecionados. Todas as 15 cepas foram resistentes à vancomicina, duas para rifampicina, uma para gentamicina e uma para o cloranfenicol. Em relação aos genes relacionados com virulência, 19 dos 49 genes testados estavam presentes em algumas cepas. Após a caracterização do potencial virulento das 15 BAL, estas foram avaliadas quanto ao potencial tecnológico para aplicação na indústria de laticínios. Todas as cepas apresentaram capacidade de acidificação, atingindo valores de pH entre 0.73 e 2.11 em 24 horas: *Lb. casei* MRUV6 apresentou maior capacidade de acidificação (pH 2.11 após 24 h). Dez cepas foram capazes de produzir diacetil a 37 °C, com exceção de *Lb. casei* MSI1, *Lb. harbinensis* MSI3, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5 e *W. paramesenteroides* MRUV3. Todas as cepas foram capazes de produzir exopolissacarídeos, e apenas duas cepas apresentaram atividade proteolítica (*Lb. casei* MSI5 e *W. paramesenteroides* MSAV5). Com base nessa caracterização, *Lb. casei* MRUV6 foi selecionado para produzir o leite fermentado, armazenado a 4 °C e 10 °C e monitorado até 35 dias de vida útil. As amostras foram submetidas a métodos fenotípicos e moleculares para avaliar a presença de *Lb. casei* MRUV6 (plaqueamento convencional e RT-PCR, verificando a expressão de *gapdh*, um gene *housekeeping*) e verificar a expressão do gene *bsh*, relacionado à resistência à sais biliares (RT-PCR). A população de *Lb. casei* MRUV6 se apresentou estável durante todo o período de armazenamento a 4 °C e 10 °C a níveis em torno de 9.9 log UFC/g e também pelo monitoramento da expressão do controle endógeno GAPDH. No entanto, o gene *bsh* não foi expresso durante o período de armazenamento. O estudo demonstrou o potencial uso da cepa de *Lb. casei* MRUV6 isolada de um ambiente lácteo para a produção de um produto lácteo fermentado e sua estabilidade durante o armazenamento a 4 °C e 10 °C. Todos os isolados do estudo apresentaram características benéficas, segurança para utilização em alimentos e potencial tecnológico para utilização na indústria de laticínios. Além disso, os mesmos podem ainda ser submetidos a estudos adicionais para avaliações *in vivo* e realizar a caracterização como probióticos.

ABSTRACT

COLOMBO, Monique, D.Sc., Universidade Federal de Viçosa, June, 2017. **Beneficial properties of lactic acid bacteria naturally occurring in dairy production environment.** Advisor: Luis Augusto Nero. Co-advisors: Svetoslav Dimitrov Todorov and Antônio Fernandes de Carvalho.

Lactic acid bacteria isolated from dairy environment were evaluated for beneficial potential. Preliminary screening and PCR analysis were applied to select and identified through 16s rRNA sequencing 15 LAB strains: *Lactobacillus* (n = 11; *Lb. casei* MSI1, *Lb. casei* MSI5, *Lb. casei* MRUV1, *Lb. casei* MRUV6, *Lb. acidophilus* MVA3, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSI3, *Lb. harbinensis* MSIV2, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5 and *Lb. plantarum* MSI2), *Pediococcus* (n = 2; *P. pentosaceus* MLEV8 and *P. acidilactici* MSI7) and *Weissella* (n = 2; *W. paramesenteroides* MRUV3 and *W. paramesenteroides* MSAV5). All selected strains showed resistance to acidic pH and to presence of bile salt. API ZYM test characterized enzymatic activity of the strains and high β-galactosidase activity was observed in 13 strains. All strains presented high values for survival rate to simulated gastric and intestinal conditions, ability to auto and co-aggregate with indicators microorganisms and high cell surface hydrophobicity. Most of the strains were positive for *map* and *EFTu* beneficial genes. Strong bile salts deconjugation was applied for all strains and all strains showed good results for assimilating lactose. After this first part of the study, the 15 BAL were evaluated for potential virulence and antimicrobial resistance. The production of virulence factors (hemolysis, gelatinase, lipase, deoxyribonuclease and biogenic amines: lysine, tyrosine, histidine and ornithine) was assessed by phenotypic methods at 25 °C and 37 °C, as well as the resistance to 17 antimicrobials. The isolates were also subjected to PCR to identify the presence of 49 genes associated with virulence factors. None of the strains presented hemolytic activity or the production of gelatinase, lipase, deoxyribonuclease and tested biogenic amines. Of the 15 selected cultures, for 12 types of antibiotics in the disc diffusion method, all strains were resistant for oxacillin and sulfa/trimethoprim, 14 were resistant to gentamicin, 11 were resistant to clindamycin, nine strains were resistant to vancomycin, eight strains to rifampicin, five were resistant to erythromycin, four were resistant to tetracycline, two strains were resistant to ampicillin, one strain was resistant to chloramphenicol and none was resistant for imipenem. For a quantitative test of the antibiogram, five antibiotics were selected in Etest® strips (bioMérieux). All 15 strains were resistant to vancomycin, two for rifampicin, one for gentamicin and one for chloramphenicol. Regarding the

virulence related genes, 19 genes from 49 tested were present in some strains. Results showed that five cultures showed the presence of the *int* gene, four cultures showed the presence of the *ant(4')-Ia* gene, three cultures were positive for *vanC2*, *cpd* and *tdc*, two cultures for *vanA, tet(K), tet(S), ermA, bcrR, mur-2ed, asa1* and *ccf,* and one culture was positive for *vanC1, ermB, aph(3')-IIIa, aac(6')-le-aph(2'')-Ia, bcrB* and *hyl.* After characterizing the virulent potential of the 15 BAL, these strains were evaluated for the technological potential for application in the dairy industry. All strains presented acidification capacity, reaching pH values between 0.73 and 2.11 in 24 hours: *Lb. casei* MRUV6 presented the highest acidification ability (pH 2.11 after 24 h). Ten strains were able to produce diacetyl at 37 °C, except by *Lb. casei* MSI1, *Lb. harbinensis* MSI3, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5 and *W. paramesenteroides* MRUV3. All strains were able to produce exopolysaccharides, and only two strains presented proteolytic activity (*Lb. casei* MSI5 and *W. paramesenteroides* MSAV5). Based on this characterization, *Lb. casei* MRUV6 was selected for producing fermented milk, stored at 4 °C and 10 °C and monitored until 35 days of shelf life. Samples were subjected to phenotypical and molecular methods to quantify the presence of *Lb. casei* MRUV6 (conventional plating and RT-PCR, by checking the expression of *gapdh*, a housekeeping gene) and to verify the expression of *bsh* gene, related to resistance to bile salts (RT-PCR). *Lb. casei* MRUV6 population was stable during storage period at 4 and 10 °C at levels around 9.9 log CFU/g, and by monitoring the expression of *gapdh* gene. However, *bsh* gene was not expressed during storage period. The study demonstrated the potential use of the beneficial strain *Lb. casei* MRUV6 isolated from a dairy environment for the production of a fermented milk product, and its stability during storage at 4 and 10 °C. All isolates from the study presented beneficial characteristics, safety for use in food and technological potential for use in the dairy industry. In addition, they may further be subjected to further studies for *in vivo* evaluations and characterization as probiotics.

INTRODUCTION

Probiotics are defined as live micro-organisms that, when ingested in adequate amounts, confer a health benefit to the host. Most of the probiotics used by the food industry are bacteria that belong to the lactic acid bacteria (LAB) group and these are considered important because of their essential role in nutrition and food industry. Fermented food products are the most used for this implication. Many products are considered vehicle for administration, however fermented dairy products are the main products for this use because of maintain optimal concentrations for consumers and there is a long history of safe use.

Probiotic cultures have been isolated from raw and fermented dairy products (such as cheese), breast milk, the oral cavity and gastrointestinal tract of humans and other animals, faecal samples of healthy adults and infants, breast-fed infant faeces, guts of several animal species (pigs, rats and poultry, for example), bee gut, intestinal tracts of marine and fresh water fish and many others. They are also found in meat and fruits, green table olives, pickled juice, wine and plant materials (e.g., wine, silage, pickles, and kimchi). In addition, scientific evidence has strengthened the dairy production chain for isolation of these cultures.

The present study has a direct interface with human health and dairy industry interest as strong innovative feature, seeking to exploit beneficial potential cultures naturally present in milk production environment. The aim of this study is perform the beneficial, safety and technological characterization of lactic acid bacteria isolated from dairy environment and characterized the first screening for potential probiotic bacteria.

OBJECTIVES

CHAPTER 1 - POTENTIAL USE OF BENEFICIAL BACTERIA BY THE DAIRY INDUSTRY.

Objetive: This review aims to elucidate important probiotic research regarding the isolation and characterization of beneficial cultures, and demonstrate the relevance of the dairy chain as a potential source of novel cultures and for the development of new probiotic products that can be considered as vehicles for them and expand the dairy industry.

CHAPTER 2 - BENEFICIAL PROPERTIES OF LACTIC ACID BACTERIA NATURALLY PRESENT IN DAIRY PRODUCTION ENVIRONMENT.

Objective: The aim of the study was to explore the dairy production environment as a source of lactic acid bacteria strains with probiotic potential.

CHAPTER 3 - VIRULENCE POTENTIAL AND ANTIMICROBIAL RESISTANCE OF BENEFICIAL LACTIC ACID BACTERIA ISOLATED FROM DAIRY PRODUCTION ENVIRONMENT.

Objective: The present study aimed to assess the virulence potential and antimicrobial resistance of the 15 lactic acid bacteria strains, in order to evaluate for safety usage by the food industry.

CHAPTER 4 - TECHNOLOGICAL PROPERTIES OF LACTIC ACID BACTERIA OBTAINED FROM A DAIRY PRODUCTION ENVIRONMENT AND DEVELOPMENT OF A FERMENTED MILK WITH THE BENEFICIAL STRAIN *Lactobacillus casei* MRUV6.

Objective: The present study aimed to characterize the technological properties of lactic acid bacteria strains isolated from a dairy production environment, previously characterized as beneficial, and to select a strain candidate to be considered as starter culture in the production of fermented milk and monitored during the shelf-life.

CHAPTER 1 - THE POTENTIAL USE OF PROBIOTIC/BENEFICIAL BACTERIA BY THE DAIRY INDUSTRY

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Title page

The potential use of probiotic/beneficial bacteria by the dairy industry

Run title: Probiotic bacteria in dairy industry

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Abstract

Scientific studies demonstrate the importance of intestinal microbiota to human health, and how probiotic microorganisms can positively affect health when administered regularly and in adequate amounts. Probiotic bacteria are can be part of fermented products and their functional importance is associated mainly with their physiological characteristics, such as their substrate utilization and metabolite production capacities. Some starter cultures can also have important health modulating and probiotic properties. Currently, the role of probiotics in improving health status is their most important use. They are thought to benefit healthy individuals using these microorganisms to maintain their health and also to strengthen resistance against various types of diseases. The acceptance of probiotic cultures and products by consumers was increased when these bacteria were marketed as natural cultures that help in digestion and health. Considering these evidences, the food industry has an increasing demand for new candidates as probiotic cultures, and the dairy industry has a particular interest for that: fermented milks and other dairy products are the most common food vehicles for probiotic cultures. Therefore, the dairy industries are increasingly seeking for improving their products with these beneficial bacteria. Future prospects show that probiotic foods represent a considerable expansion of the dairy industry, allowing the dairy sector to grow and to be increasingly sought by the consumer to maintain their health based on a diet supplemented with probiotic foods all over the world. This review aims to elucidate important probiotic research regarding the isolation and characterization of beneficial cultures, and demonstrate the relevance of the dairy chain as a potential source of novel cultures and for the development of new probiotic products that can be considered as vehicles for them and expand the dairy industry in Brazil.

Keywords: Probiotics, dairy environment isolation, lactic acid bacteria, beneficial properties, dairy industry.

Introduction

Probiotics are defined as living organisms that benefit consumer health when ingested in appropriate concentrations (FAO/WHO, 2002). To date, most of the probiotics used by the food industry are bacteria that belong to the lactic acid bacteria (LAB) group and these are considered important because of their essential role in nutrition and food (Kechagia et al., 2013). They are used in fermented food products and their beneficial/functional importance is associated mainly with their physiological characteristics, such as their substrate utilization and metabolite production capacities, as well as their important probiotic properties (Liu et al., 2011; Oh & Jung, 2015). The acceptance of probiotic cultures and products by patient and/or consumers was increased when these bacteria were marketed as natural cultures that help in digestion processes and health (Kechagia et al., 2013). The development of probiotic food products can affect a large part of the population, however therapeutic applications of LAB have a more limited scope (Foligné et al., 2013).

There are several beneficial properties associated with probiotic microorganisms. Among their benefits are: antimutagenic, anticarcinogenic, antioxidant, and antidiarrheal properties; as well as activities concerning stimulation of the immune system, prevention of eczema and atopic dermatitis, lowering of blood pressure, reduction in the concentration of cholesterol, resistance to infectious diseases, growth stimulation, control of gastrointestinal inflammatory diseases, maintenance of balanced microbiota and improvement of lactose metabolism. Currently, the role of probiotics in improving health is the most important characteristic, where it is expected that healthy individuals can use these microorganisms to maintain their health and as protection against different types of diseases (Amara & Shibl, 2015).

This review aims to elucidate important research regarding the isolation and characterization of probiotic cultures use in food industry and human consumption.

Healthy lifestyle and good nutrition

Health and welfare form part of the desires of the world population majority. The search for quality of life and well-being is a concern that is progressively present throughout society, which is reflected in people life. Good nutrition and good quality of life are part of the same lifestyle philosophy and it is fundamental to the well-being and economic and social development of a country (Kechagia et al., 2013). Nutrition is a

fundamental health factor and its association with fitness activities is essential for health promotion and disease prevention (Wakeman, 2013; Ouwehand & Roytio, 2014; Cox et al., 2014).

The link between nutrition from the diet and diseases is well documented and there are several studies that prove that what we eat has a huge impact on how we feel. Our food can significantly influence the composition and function of the intestinal microbiota, thus influencing our health (Ceapa et al., 2013; Amara & Shibl, 2015; Salmerón et al., 2015). Increasingly, scientific evidences demonstrated the importance of intestinal microbiota to human health: probiotic microorganisms can positively affect host health when administered regularly and in adequate amounts (FAO/WHO, 2006; Kechagia et al., 2013).

Strains classified as probiotic bacteria are considered to be important with regard to the food and nutrition of humans and other animals. These cultures are present in dairy production chain: that ranges from milk production, the milk and technology employed, through the processing plants, the product shelf life on retail stores and the supply to the end consumer (Rodríguez et al., 2000). Scientific evidence has strengthened the dairy production chain, leading to an increasing interest in, and production by the market, of functional foods. The growing consumer awareness about the relationship between health and nutrition creates a supportive environment for the development of functional products. They benefit the health of consumers as well as providing nutritional value, as in the case of probiotic products (Kechagia et al., 2013).

Definition of probiotic microorganisms

According to FAO/WHO (2002) probiotics are defined as living organisms that, when ingested in adequate concentrations, benefit consumer health. Several studies have been developed to characterise the probiotic activity of these microorganisms as well as their applications in the production of traditional and commercial fermented foods. According to the Scopus database (www.scopus.com, accessed 28 May 2016), more than 25,802 articles related to probiotics have been published since 1954, including 5,339 review articles (SCOPUS, 2016). Over the past 20 years, research in this area has progressed considerably. There have been significant advances in the selection and characterization of probiotic cultures, focusing on benefits to consumer health (FAO/WHO, 2001). Probiotic microorganisms are represented by different genera and including bacteria, yeasts and moulds. Among the most common probiotic bacteria are:

1) *Lactobacillus: Lb. acidophilus, Lb. sporogenes, Lb. plantarum, Lb. rhamnosus, Lb. delbrueckii, Lb. reuteri, Lb. fermentum, Lb. lactis, Lb. cellobiosus, Lb. brevis, Lb. casei, Lb. farciminis, Lb. paracasei, Lb. gasseri, Lb. crispatus, Lb. mucosae;* 2) *Bifidobacterium: B. bifidum, B. infantis, B. adolescentis, B. longum, B. thermophilum, B. breve, B. lactis, B. animalis;* 3) *Streptococcus: S. lactis, S. cremoris, S. salivarius, S. intermedius, S. thermophilus, S. diacetylactis;* 4) *Leuconostoc mesenteroides;* 5) *Pediococcus;* 6) *Propionibacterium;* 7) *Bacillus; 8*) *Enterococcus: E. faecium, E. mundtii* (Amara & Shibl, 2015; Kechagia et al., 2013). *Bifidobacterium* and *Lactobacillus* are the most widely used in probiotic products (Holzapfel et al., 2001; Fontana et al., 2013).

LAB are commonly used in fermented food products and their importance is mainly associated with their physiological characteristics, such as their use of different substrates, their metabolic capabilities and their health promoting properties (Oh & Jung, 2015). They are characterised by their organoleptic characteristics, the determination of their products and their interference in the survival and detection of foodborne pathogens. These bacteria are also known for their ability to produce substances with antimicrobial activity against spoilage and pathogenic microorganisms (Dal Bello et al., 2010; Guinane et al., 2005; Rodríguez et al., 2000).

Probiotics may affect a large part of the population as nutritional supplements, while therapeutic applications have a narrower range (Foligné et al., 2013). Acceptance of probiotics by consumers became mainstream when the bacteria were marketed as natural cultures that aid in digestion and health. While most probiotics currently belong to food-grade species, in the future may new microorganisms may be used in functional foods and pharmacology (Foligné et al., 2013).

Increasingly, probiotics have been sought in different food systems, making it a growing trend. However, the search for new probiotic sources may lead to a reduction in the efficacy of the probiotic culture due to the lack of synergism between the food and the probiotic bacteria (Boza-Méndez et al., 2012). Thus, the selection of a suitable food matrix for the addition of a LAB and the production of a probiotic food becomes an important factor for the development of functional products (Boza-Méndez et al., 2012; Ranadheera et al., 2010).

Dairy industries are often seeking for novel probiotic strains, in order to develop novel dairy products to be offered to consumers as alternatives to keep their health and well-being. Nowadays, the ability to innovate is mandatory for the food industry, including the dairies. In this context, the development of new probiotic products with

the addition of new strains characterized as beneficial is increasingly challenging for the food industry, as beyond the functionality of the product is the expectation of consumers for ever better products (Granato et al., 2010). As an example of new probiotic foods, chocolate desserts can be highlighted, which allow the development of new probiotic products with economic potential (Rosa et al., 2016), and also fresh and soft cheeses in Brazil, in which are added cultures with simultaneous technological, beneficial and safety properties (dos Santos et al., 2015).

Beneficial effects of probiotic microorganisms

Human individuals can be exposed to different types of conditions that are detrimental to health. For example, stress, the use of antibiotics, inadequate food (including diets high in fat, meat, salt and sugar), excessive alcohol use, exposure to chlorine and fluoride in drinking water, exposure to environmental toxins and many other factors could influence and change the intestinal microbiota balance. These factors are responsible for the development of diseases and probiotics should be used for microbiota regeneration and health promotion (Amara & Shibl, 2015).

Currently, the role of probiotics in improving health is their most important function, where healthy individuals use these microorganisms to further improve their health and also to protect against various types of diseases (Amara & Shibl, 2015). Probiotics mainly have gastrointestinal applications, however their use can be easily extended to the skin, oral, and vaginal health (Foligné et al., 2013). However, these latter probiotic applications should be considered with a moderate dose of scepticism, since according to the World Health Organisation (WHO) probiotics are life organisms that present beneficial effects on the gastrointestinal tract. In the last decade reports have been showing that even dead cells, or cell parts, can have a positive effect on the immune system and other similar effects can be attributed to probiotics. Some authors use terms such as “pharma biotics”, “beneficial” or other words to describe these “probiotics” that do not fit the WHO definition. Maybe a new, updated definition of probiotics will be requested in the near future in order to avoid misunderstanding and to provide a proper classification and definition of this group of organisms. Evidence-based studies have shown the potential clinical effectiveness of these bacteria in the prevention and treatment of many diseases (Fontana et al., 2013). In addition to the maintenance of health and the control of pathogenic infections, scientific studies have shown that probiotics are promising for the control and direct treatment of specific

diseases, and can be directly involved in prophylaxis and prevent active medical practices (Amara & Shibl, 2015; Fontana et al., 2013).

Little is known about how probiotics influence host intestinal microbiota and therefore the mechanisms for their beneficial effects are difficult to determine (Gibson & Fuller, 2000). Although the mechanisms of action of these microorganisms are not yet completely understood, studies show the growing scientific evidence for their health benefits and safety for human consumption (Aguirre-Ezkauriatza et al., 2010; Ahire et al., 2013). Mechanisms of action can include proteins and short chain fatty acid production, lowering of gut pH and nutrient competition that stimulates mucosal barrier function and immunomodulation. Immunomodulation has been the most studied and this action is verified by the capacity of probiotics to induce phagocytosis and IgA secretion, modifying T-cell responses, enhancing Th1 responses, and attenuating Th2 responses (Kechagia et al., 2013). In a general sense, the mechanisms of action of probiotics are multi-factorial: gut microbiota modification, competitive adherence to the mucosa and epithelium, gut epithelial barrier strengthening and immune system modulation all convey advantages to the host (Fontana et al., 2013).

It is important to remember that to understand the mechanisms by which probiotic microorganisms are able to assist in the control and treatment of diseases, it is also necessary to understand the mechanism of the disease and its causative agents. It should also be borne in mind that probiotic effects tend to be strain specific: each strain can bring different benefits to the host (Fontana et al., 2013; Amara, 2013). For example, genetically inherited lactose intolerance causes certain types of disability to the body. Probiotics could act by different mechanisms to mitigate such shortcomings: supplying products that the body is not able to provide via missing genes; providing adequate alternative products; providing end products of the complete pathway (for single or multiple gene deficiencies that are blocked in certain pathways); support a different pathway in which there is no defect in either alleles; enable load reduction on our biological system perhaps by allowing an extra activity (e.g. in the case of utilisation of nutrients) (Amara, 2013). Figure 1 shows the mechanisms of action of probiotic bacteria.

There are several benefits of probiotic bacteria. Many studies support the use of probiotics benefits for prevention, control and treatment of diseases as well as general health maintenance. Figure 2 represents the benefits to the host organism for which probiotics are responsible.

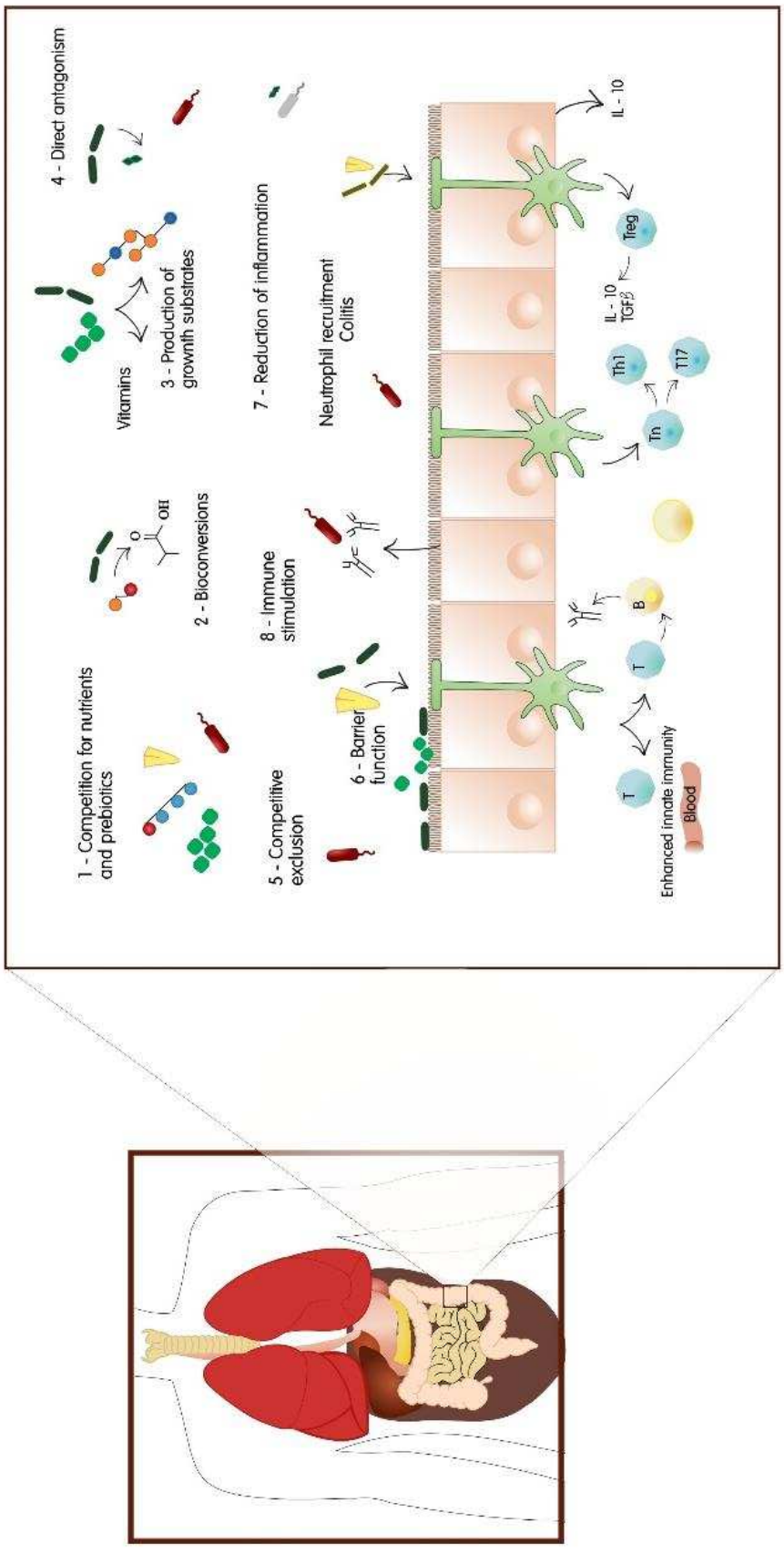

Figure 1. Probiotic action mechanisms.

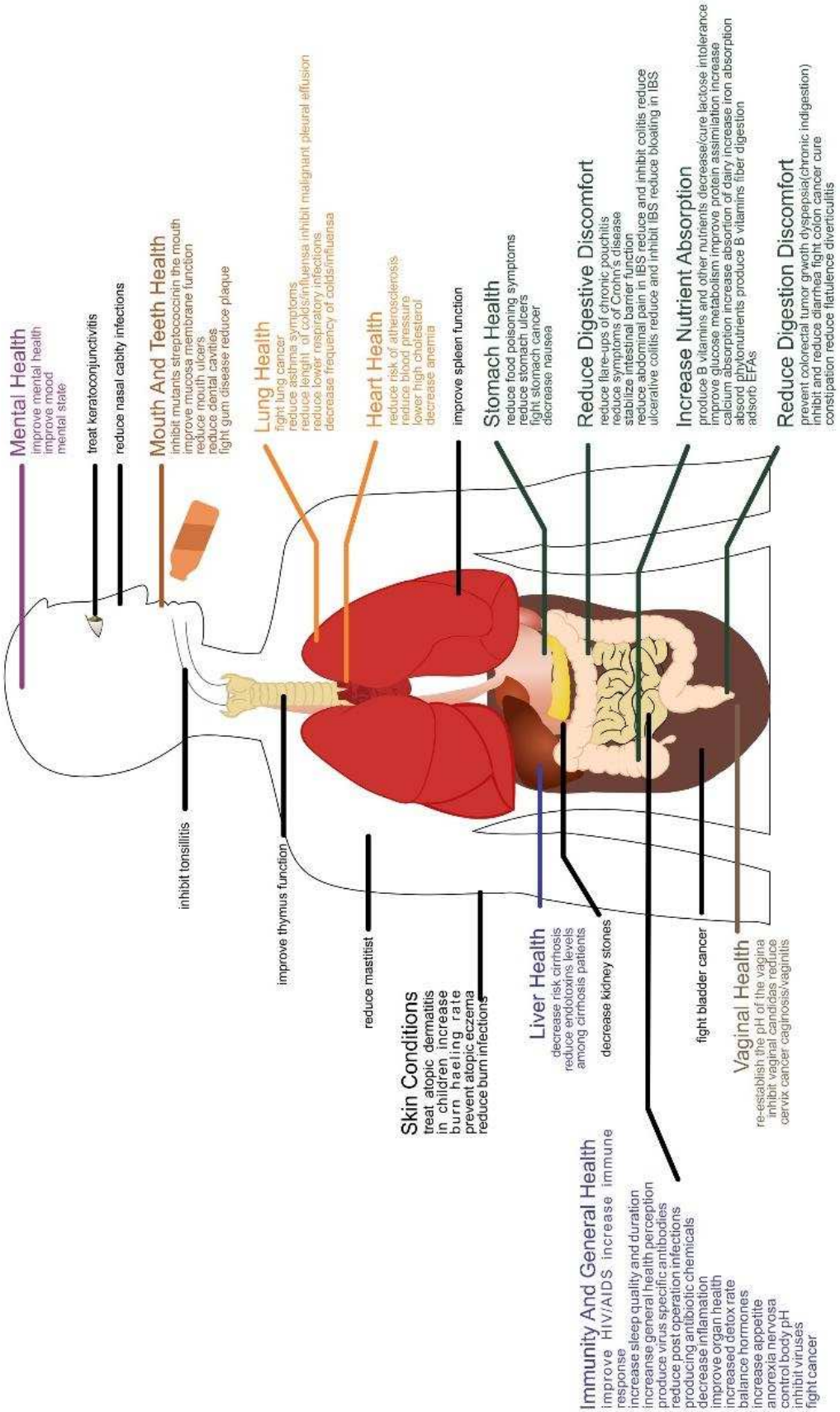

Figure 2. Beneficial effects of probiotic bacteria.

Probiotic selection criteria

The criteria for selection of new probiotic species include features related to safety and functionality, as well as technological aspects of the cultures to be used in the product composition and tests in animal models and *in vivo* tests (Gueimonde & Salminen, 2006; FAO/WHO, 2006). With regard to the safety of these microorganisms, they must come from healthy animals, be normal bowel inhabitants and be non-toxic and non-pathogenic (Salminen et al., 1999). The functionality is related to the ability to withstand the harsh conditions of the gastrointestinal tract (action of bile, gastric, pancreatic and enteric juices), as well as possession of antagonistic activity against resident pathogens (Cotter et al., 2005; Salminen et al., 1999; Servin, 2004). The required technological properties are: easy manipulation, fast growth *in vitro*, stability during storage (surviving in the final product and retaining function) and ability to multiply along with autochthonous microbiota of the host (Salminen et al., 1999; FAO/WHO, 2002). Finally, animal models and *in vivo* tests are fundamental to the culture definition as probiotic.

Probiotic cultures have been isolated from raw and fermented dairy products (such as cheese), breast milk, the oral cavity and gastrointestinal tract of humans and other animals, faecal samples of healthy adults and infants, breast-fed infant faeces, guts of several animal species (pigs, rats and poultry, for example), bee gut, intestinal tracts of marine and fresh water fish and many others. They are also found in meat and fruits, green table olives, pickled juice, wine and plant materials (e.g., wine, silage, pickles, and kimchi) (Fontana et al., 2013; Broadbent et al., 2012; Su et al., 2015). In addition, scientific evidence has strengthened the dairy production chain for isolation of these cultures.

There are several important selection criteria for the success of probiotics and they are shown in Table 1. Not all probiotic possess all these features, but it is desirable that they presents as many of them as possible (Gueimonde & Salminen, 2006; FAO/WHO, 2006).

Table 1. Selection criteria for the success of probiotics.

Criteria items	Description
Strain source	Isolates from the same host species as the intended use have a greater chance of survival.
Safety properties	Probiotics should be recognised as GRAS (generally Recognized as safe) with minimal possibility of antimicrobial resistance transfer; they should be safe for food use with proven health promoting effects (non-invasive in epithelial cell line models, production of anti-inflammatory rather than proinflammatory cytokines, no antibiotic resistance).
Survival	Either on the product, or after ingestion thereof. Strains with greater resistance to acid pH, bile secretions and those capable of adhering to the intestinal epithelium have stronger survival characteristics
Production characteristics	Ability to grow in mass without genetic variation
Processing	Enough strength to withstand the rigours of incorporation in oral delivery systems
Sensory properties	When added in food, product quality should not decrease
Microbiological properties	Ability to survive in the gastrointestinal tract microbial ecosystem
In vivo tests	Animal models and human tests
Effects on consumers	No adverse side effects. Effects on intestinal transit should occur
Adhesion	To increase the survival in the gut, balancing intestinal microbiota, intestinal permeability, local microecology, alleviation of inflammation, barrier strengthening
Effects on pathogens	Many probiotics are able to inhibit harmful microorganisms by the production of acid, bacteriocins or competitive exclusion
Metabolic activity modulation and metabolite production	Inactivation of pro-carcinogens, normalisation of barrier function, barrier strengthening, regulating bowel movements
Immunomodulation	Probiotics should affect the system so that less pathogen resistance occurs, as well as positive aspects regarding food allergy
Cytokine production	Protecting against deviations in intestinal immune responses
Assessing toxin binding	Protecting the intestinal integrity and reducing the risk of contaminant induced deviations
Impact on gene expression	Positive health effects locally on target tissues and reduction in disease risk
Genomic information	Specific data on probiotics and selection based on host properties

Despite the great interest of the industry in using probiotic cultures in food products to impart benefits to consumers, the use of these strains requires great caution because of the possibility and possessing antimicrobial resistance genes and transferring them to other microorganisms (Barbosa et al., 2010). With regard to food safety, selecting a probiotic culture must be performed very carefully (FAO/WHO, 2001). Strains that possess virulence genes can express them in food, presenting a risk to consumers (Moraes et al., 2012). Probiotic microorganisms may also have resistance to different antibiotics and carry genes related to these characteristics, increasing their potential virulence (Ammor et al., 2007). The possibility of vertical transfer of genes between probiotic cultures and other bacteria is a concern in the food industry (Toomey et al., 2010).

With the progress of molecular biology, the identification and differentiation of bacterial strains has become more sensitive and reliable. Classical microbiological techniques are very important to the selection, enumeration and biochemical characterization of the strains. However, they are not sufficient to classify taxonomically cultures. Molecular methodologies such as polymerase chain reaction (PCR) and sequencing of specific genes conserved among genera and species are the most potent and precise methods to identify isolates of interest.

In recent years, a wide variety of molecular techniques based on the study of the similarity of the chromosomes has been developed, especially Pulsed-Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST). MLST is a molecular characterisation technique in epidemiological research, being widely used for phylogenetic inference of bacterial species; one of its major advantages is that it is based on DNA sequence and not on electrophoretic methods, which allows a more precise analysis (Maiden et al., 1998). Regarding PFGE, the technique is based on the separation of DNA fragments of high molecular weight, obtained by digestion of bacterial genomic DNA with restriction enzymes (FAO/WHO, 2006). RAPD and Rep-PCR are other molecular techniques for genetic identification of isolates that are also important for the characterisation of isolated genetic profiles, allowing the selection of strains with desired characteristics (Dal Bello et al., 2010).

Dairy products as vehicles for probiotic cultures

According to Ashraf and Shah (2011), more than 500 probiotic products were introduced into the market worldwide between 2000 and 2010. Since then, several

studies have shown the health benefits and safety of probiotic microorganisms (Aguirre-Ezkauriatza et al., 2010; Maragkoudakis et al., 2006). Thus, there is great commercial interest in developing new probiotic food products. Probiotic cultures have fuelled widespread applications in the food and health industries due to their broad ecological distribution that reflects their metabolic flexibility (Broadbent et al., 2012). Since the 1990s, fermented milk products have received significant attention because of the presence of these probiotic microorganisms in their composition. Fermented milk products are considered the main vehicle for administration at optimal concentrations for consumers and there is a long history of safe use (Karimi et al., 2012; Fontana et al., 2013). These products are marketed as functional foods mainly in Europe, Japan, the USA and Australia (Phillips et al., 2006).

Functional foods promote beneficial effects beyond their basic nutritional components, such as probiotics (Heasman & Mellentin, 2001). The Japanese government was one of those responsible for the description of probiotic products, introducing the term “foods for specified health use” (FOSHU) and in order to reduce spending on public health, separating functional foods from pharmaceuticals (Sanders & Huis in't Veld, 1999). In the same context, the scientist Minoru Shirota, through the sale of the fermented milk drink Yakult, put into practice the concept that disease prevention is better than treatment, and that a healthy intestine leads to a long life. Thus, *Lb. casei* Shirota is highlighted as the first commercially presented probiotic microorganism, possessing a strong probiotic appeal.

To exploit consumers’ quest for a healthier life style, food manufacturers have used various probiotic strains combined in the same product, in addition to starter cultures inoculated in the production of fermented foods (Kechagia et al., 2013; Cox et al., 2014). Fermented milks are considered the main vehicles for probiotic microorganisms (Ranadheera et al., 2010). These products have always been recognized by consumers for their many desirable effects (Lourens-Hattingh & Viljoen, 2001). Moreover, they have greater nutritional benefits than milk itself, being nutritionally richer in protein, calcium, riboflavin, and vitamins B6 and B12 (Ashraf & Shah, 2011). Starter cultures are employed to promote the modifications in the food matrix resulting in the fermented product, and are usually not able to survive passage through the intestinal tract, enabling the consumer to enjoy their probiotic potential. As an alternative, there is a growing trend for adding other probiotic cultures in the same product, such as *Lb. casei* (Ashraf & Shah, 2011).

Additionally, other dairy products are commonly used as carriers of probiotic cultures, for example fresh cheese whey and its by-products. Several studies have shown the potential of these products that retain the beneficial features of the added cultures with direct effects on consumer health (Esmerino et al., 2013; Lollo et al., 2013).

In addition, there is a trend to seek viable alternatives in the processing of probiotic products, ensuring the viability and shelf life of products: high-pressure (HP) processing and pulsed electric fields (PEF) promise to guarantee these characteristics and maintain product functionality. Both processes are emerging and would help maintain the beneficial properties of probiotics, since traditional thermal techniques are capable of destroying the beneficial cures added in probiotic products (da Cruz et al., 2010).

Future prospects: expansion of the dairy industry through scientific research

Functional foods, including probiotic foods, have increased significantly from the results and scientific advances in research with these foods in recent years. In addition, the consumer has been based on the knowledge about the relationship of certain foods and the benefits that the same provides through their consumption to make their food choices. For these and several other reasons, probiotic foods represent a relevant expansion of the dairy industry allowing the dairy sector to grow and be increasingly sought by the consumer to maintain their health based on a diet supplemented with probiotic foods. Increasingly new products have emerged as attractive alternatives by the addition of beneficial bacteria by the dairy industry. More and more research has shown the beneficial value of these isolated cultures from different environments, the nutritional value of dairy products and the excellent result between food and the addition of probiotic bacteria in this product.

The diversity of dairy products in Brazil and around the world varies considerably between regions, and the reasons for this diversity are explained by the dietary habits, types of dairy processing technologies used, the market demand available, and social and cultural circumstance (IFCN, 2013, FAO, 2017). In developed countries, per capita consumption of dairy products is higher, but this gap is decreasing as developing countries begin to make a significant share of this consumption. This increase in consumption by developing countries occurs because of increased income, population growth, urbanization and changes in diets and eating habits (FAO, 2017).

Populated countries such as China, Indonesia and Vietnam, as well as Asian regions, are part of this trend in increased consumption of dairy products (IFCN, 2013, FAO, 2017). The growing demand for dairy products has led to a growing expansion of both the dairy industry and the producers, who are responsible for providing quality raw material and which can increase production and profitability. Fluid milk is the most widely consumed dairy product in the world, yet dairy products are becoming increasingly important in the population consumption of many countries (FAO, 2017).

Many researches have shown the importance of probiotic bacteria added in dairy products to human health as well as the nutritional part of these quality foods inserted into the diet of consumers as healthy options in dairy have significantly outperformed the rest of the dairy sector in recent years (Brockman and Beeren, 2011). In Brazil, many studies had demonstrated the presence of beneficial strains in dairy products, and have characterized them as potential isolates to be used as commercial starter cultures by the food industry.

Ramos et al. (2013) identified and characterized potential probiotic isolates from different Brazilian fermented foods: *Lb. fermentum* (34 isolates), *Lb. plantarum* (10) and *Lb. brevis* (7). Costa et al. (2013) found that artisanal cheese from Serra da Canastra can transport microorganisms with probiotic potential to consumers: *Lb. rhamnosus* B4, *W. paramesenteroides* C10 and *Lb. rhamnosus* D1 would be candidates for the elaboration of new dairy cultures for the production of potentially functional fermented dairy products, which could help food security and preservation of the original microbiota of artisanal cheeses from Minas Gerais. Jeronimo-Ceneviva et al. (2014) found that *L. casei* SJRP35, *Leuconostoc citreum* SJRP44, *Lb. delbrueckii* subsp. *bulgaricus* SJRP57 and *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP58 strains isolated from water buffalo mozzarella cheese presented different potential probiotic characteristics. Meira et al. (2015) evaluated the effects of added *Lb. acidophilus* La-05 or *B. lactis* Bb-12 probiotics on the quality characteristics of goat ricota: these probiotics did not negatively affect the general quality characteristics of this product and suggested that goat ricotta is an efficacious food matrix for maintaining the viability of these probiotics during storage and under the stressful conditions imposed by the human gastrointestinal tract. Bezerra et al. (2017) did the first work which focused primarily on the volatile profile of goat Coalho cheese added with probiotic lactic bacteria (in isolated or combined form) and they concluded that both the use of different cultures of probiotic lactic bacteria and the length of storage affect the volatile profile. Machado et al. (2017) studied the incorporation of honey produced by

the stingless bee *Melipona scutellaris* in goat yogurt containing the probiotic *Lb. acidophilus* La-05 and they concluded that positively affected some of the assessed physical and mechanical stability characteristics of the product during the 28 days of refrigerated storage; namely color, syneresis, viscosity and water retention capacity. Among many other researches that have contributed to the advancement in the study of probiotics and the expansion of the dairy industries, which increasingly seek to increment their dairy products with these beneficial bacteria.

Concluding remarks

There is no doubt about the benefits to human health of incorporating probiotics into food products. Specific probiotic treatments are also important for the prevention and control of diseases. Moreover, the increasing consumer awareness about the benefits of these microorganisms has meant that probiotic products have become increasingly popular and represent an important functional food market. Dairy products remain the most important vehicle for the administration of probiotic bacteria and increasingly these products have been incorporated into the human diet because of technological advances in this area.

Considering this, the beneficial role of probiotics in nutrition and human medicine is evident. However specific studies regarding isolation, characterization, safety and application of these microorganisms in food are necessary, as well as accurate studies on their mechanisms of action. Thus, it is very important to standardize the methods used for these studies in order to gain a better comparison of obtained research results and consequently increased reliability of the results presented.

References

Aguirre-Ezkauriatza EJ, Aguilar-Yanez JM, Ramirez-Medrano A & Alvarez MM 2010 Production of probiotic biomass (*Lactobacillus casei*) in goat milk whey: comparison of batch, continuous and fed-batch cultures. Bioresource Technology 101(8) 2837-2844

Ahire JJ, Mokashe NU, Patil HJ & Chaudhari B 2013 Antioxidative potential of folate producing probiotic *Lactobacillus helveticus* CD6. Journal of Food Science and Technology 50(1) 26-34

Amara AA 2013 The inevitability of balanced lives: genes–foods–action-interactions. IIOB Journal 4(2) 1-27

Amara AA & Shibl A 2015 Role of probiotics in health improvement, infection control and disease treatment and management. Saudi Pharmaceutical Journal 23(2) 107-14

Ammor MS, Florez AB & Mayo B 2007 Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology 24(6) 559-570

Anukam KC, Reid G & ARM C 2007 Probiotics: 100 years (1907-2007) after Elie Metchnikoff's Observation. Communicating Current Research and Educational Topics and Trends in Applied Microbiology 466-474

Ashraf R & Shah NP 2011 Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt--a review. International Journal of Food Microbiology 149(3) 194-208

Barbosa J, Gibbs PA & Teixeira P 2010 Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. Food Control 21(5) 651-656

Bezerra TKA, Arcanjo NMO, Araújo ARR, Queiroz ALM, Oliveira MEG, Gomes AMP, Madruga MS 2017 Volatile profile in goat coalho cheese supplemented with probiotic lactic acid bacteria. LWT – Food Science and Technology 76, 209-2015

Boza-Méndez E, López-Calvo R, Cortés-Muñoz M 2012 Innovative dairy products development using probiotics: challenges and limitations. Probiotics 10(1) 213-236

Broadbent JR, Neeno-Eckwall EC, Stahl B, Tandee K, Cai H, Morovic W, Horvath P, Heidenreich J, Perna NT & Barrangou R 2012 Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation. BMC Genomics 13(1) 533

Brockman C and Beeren CJM 2011 Consumer perceptions of additives in dairy products. Leatherhead Food Research, 41-48

Buriti FCA, Bedani R, Saad SMI 2016 Chapter 23 – Probiotic and prebiotic dairy desserts. Probiotics, Prebiotics, and Synbiotics - Bioactive Foods in Health Promotion, 345-360

Ceapa C, Wopereis H, Rezaiki L, Kleerebezem M, Knol J & Oozeer R 2013 Influence of fermented milk products, prebiotics and probiotics on microbiota composition and health. Best Practice & Research Clinical Gastroenterology 27(1) 139-155

Costa HHS, Souza MR, Acúrcio LB, Cunha AF, Resende MFS, Nunes AC 2013 Potencial probiótico *in vitro* de bactérias ácido-láticas isoladas de queijo-de-minas artesanal da Serra da Canastra, MG. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 65(6) 1858-1866

Cotter PD, Hill C & Ross RP 2005 Bacteriocins: developing innate immunity for food. Nature Reviews in Microbiology 3(10) 777-788

Cox AJ, West NP, Horn PL, Lehtinen MJ, Koerbin G, Pyne DB, Lahtinen SJ, Fricker PA & Cripps AW 2014 Effects of probiotic supplementation over 5 months on routine haematology and clinical chemistry measures in healthy active adults. European Journal of Clinical Nutrition 68(11) 1255-1257

Da Cruz AG, Faria JAF, Saad SMI, Bolini HMA, Sant´Ana AS, Cristianíni M 2010 High pressure processing and pulsed electric fields: potential use in probiotic dairy foods processing. Trends in Food Science & Technology 21(10) 483-493

Dal Bello B, Rantsiou K, Bellio A, Zeppa G, Ambrosoli R, Civera T & Cocolin L 2010 Microbial ecology of artisanal products from North West of Italy and antimicrobial activity of the autochthonous populations. LWT - Food Science and Technology 43(7) 1151-1159

Dos Santos KMO, Vieira ADS, Salles HO, Oliveira JS, Rocha CRC, Borges MF, Bruno LM, Franco BDGM, Todorov SD 2015 Safety, beneficial and technological properties of *Enterococcus faecium* isolated from Brazilian cheeses. Brazilian Journal of Microbiology 46(1) 237-249

Esmerino EA, Cruz AG, Pereira EPR, Rodrigues JB, Faria JAF & Bolini HMA 2013 The influence of sweeteners in probiotic Petit Suisse cheese in concentrations equivalent to that of sucrose. Journal of Dairy Science 96(9) 5512-5521

FAO/WHO 2001. Expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, Córdoba, Argentina. 1-4pp.

FAO/WHO 2002: Working group on drafting guidelines for the evaluation of probiotics in food. Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada.

FAO/WHO 2006 Health and nutritional properties and guidelines for evaluation. Food and Nutrition, Rome Paper 85

FAO/WHO 2017 Producción y productos - Leche y productos lácteos. Available in http://www.fao.org/agriculture/dairy-gateway/leche-y-productos-lacteos/es/#.WOenNYgrLIV. Acessed in 2017 april 07.

Foligné B, Daniel C & Pot B 2013 Probiotics from research to market: the possibilities, risks and challenges. Current Opinion in Microbiology 16(3) 284-292

Fontana L, Bermudez-Brito M, Plaza-Diaz J, Munoz-Quezada S & Gil A 2013 Sources, isolation, characterisation and evaluation of probiotics. The British Journal of Nutrition 109 Suppl 2 S35-50

Gibson GR & Fuller R 2000 Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. Journal of Nutrition 130(2S Suppl) 391S-395S

Granato D, Branco GF, Cruz AG, Faria JAF, Shah NP 2010 Probiotic dairy products as funcional foods. Comprehensive Reviews in Food Science and Food Safety 9(1) 455-470

Gueimonde M & Salminen S 2006 New methods for selecting and evaluating probiotics. Digestive and Liver Disease 38 Suppl 2 S242-247

Guinane CM, Cotter PD, Hill C & Ross RP 2005 Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. Journal of Applied Microbiology 98(6) 1316-1325

Heasman M & Mellentin J 2001 *The functional foods revolution: healthy people, healthy profits?* London and Sterling Earthscan Publications Ltd

Holzapfel WH, Haberer P, Geisen R, Björkroth J & Schillinger U 2001 Taxonomy and important features of probiotic microorganisms in food and nutrition. The American Journal of Clinical Nutrition 73(2) 365s-373s

International Farm Comparison Network (IFCN). 11th IFCN Supporter Conference. Kiel, Germany. 2013.

Jeronymo-Ceneviva AB, Beatriz A, Paula AT, Silva LF, Todorov SD, Franco BDGM, Penna ALB 2014 Probiotic Properties of lactic acid bacteria isolated from water-buffalo mozzarella cheese. Probiotics & Antimicromicrobial Proteins 1-16

Karimi R, Mortazavian AM & Amiri-Rigi A 2012 Selective enumeration of probiotic microorganisms in cheese. Food Microbiology 29(1) 1-9

Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N & Fakiri EM 2013 Health benefits of probiotics: a review. ISRN Nutrition 2013 481651

Klaenhammer TR 2000 Probiotic bacteria: today and tomorrow. Journal of Nutrition 130(2S Suppl) 415S-416S

Lilly DM & Stillwell RH 1965 Probiotics: growth-promoting factors produced by microorganisms. Science 147(3659) 747-748

Liu S, Han Y & Zhou Z 2011 Lactic acid bacteria in traditional fermented Chinese foods. Food Research International 44(3) 643-651

Lollo PCB, Moura CS, Morato PN, Cruz AG, Castro WF, Betim CB, Nisishima L, Faria JAF, Maróstica Junior M, Fernandes CO & Amaya-Farfan J 2013 Probiotic yogurt offers higher immune-protection than probiotic whey beverage. Food Research International 54(1) 118-124

Lourens-Hattingh A & Viljoen BC 2001 Yogurt as probiotic carrier food. International Dairy Journal 11(1) 1-17

Machado TADG, Oliveira MEG, Campos MIF, Assis POA, Souza EL, Madruga MS, Pacheco MTB, Pintado MME, Queiroga RCRE 2017 Impact of honey on quality characteristics of goat yogurt containing probiotic *Lactobacillus acidophilus*. LWT - Food Science and Technology, 80, 221-229

Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M & Spratt BG 1998 Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences USA 95(6) 3140-3145

Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B & Tsakalidou E 2006 Probiotic potential of *Lactobacillus* strains isolated from dairy products. International Dairy Journal 16(3) 189-199

Meira QGS, Magnani M, Medeiros Júnior FC, Queiroga RCRE, Madruga MS, Gullóne B, Gomes AMP, Pintado MME, Souza EL 2015 Effects of added *Lactobacillus acidophilus* and *Bifidobacterium lactis* probiotics on the quality characteristics of goat ricotta and their survival under simulated gastrointestinal conditions. Food Research International 76, 828–838

Moraes P, Perin L, Todorov SD, Silva A, Franco B & Nero L 2012 Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese. Journal of Applied Microbiology 113(2) 318-328

Nanno M, Kato I, Kobayashi T & Shida K 2011 Biological effects of probiotics: what impact does *Lactobacillus casei Shirota* have on us? International Journal of Immunopathology and Pharmacology 24(1 Suppl) 45S-50S

Oh YJ & Jung DS 2015 Evaluation of probiotic properties of *Lactobacillus* and *Pediococcus* strains isolated from Omegisool, a traditionally fermented millet alcoholic beverage in Korea. LWT - Food Science and Technology 63(1) 437-444

Ouwehand AC & Roytio H 2014 *Probiotic fermented foods and health promotion.* Elsevier

Phillips M, Kailasapathy K & Tran L 2006 Viability of commercial probiotic cultures (*Lb. acidophilus, Bifidobacterium* sp., *L. casei, L. paracasei* and *L. rhamnosus*) in cheddar cheese. International Journal of Food Microbiology 108(2) 276-280

Ramos CL, Thorsen L, Schwan RF, Jespersen L 2013 Strain-specific probiotics properties of *Lactobacillus fermentum, Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. Food Microbiology 36, 22-29

Ranadheera R, Baines S & Adams M 2010 Importance of food in probiotic efficacy. Food Research International 43(1) 1-7

Rodríguez E, González B, Gaya P, Nuñez M & Medina M 2000 Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. International Dairy Journal 10(1–2) 7-15

Rosa LJB, Esper LMR, Cabral JPLG, Franco RM, Cortez MAS 2016 Viability of probiotic micro-organism *Lactobacillus acidophilus* in dairy chocolate dessert and its action against foodborne pathogens. Ciência Rural 46(2) 368-374

Salmerón I, Thomas K & Pandiella SS 2015 Effect of potentially probiotic lactic acid bacteria on the physicochemical composition and acceptance of fermented cereal beverages. Journal of Functional Foods 15 106-115

Salminen S, Ouwehand A, Benno Y & Lee YK 1999 Probiotics: how should they be defined? Trends in Food Science & Technology 10(3) 107-110

Sanders ME & Huis in't Veld J 1999 Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie Van Leeuwenhoek 76(1-4) 293-315

SCOPUS 2016 Research tool: probiotics. In Book Research tool: probiotics. Series Research tool: probiotics. Elsevier.

Servin AL 2004 Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiology Reviews 28(4) 405-440

Su J, Wang T, Li YY, Li J, Zhang Y, Wang Y, Wang H & Li H 2015 Antioxidant properties of wine lactic acid bacteria: *Oenococcus oeni*. Applied Microbiology and Biotechnology 99(12) 5189-5202

Toomey N, Bolton D & Fanning S 2010 Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. Research in Microbiology 161(2) 127-135

Wakeman M 2013 A review of the role of probiotics in sport. British Journal of Sports Medicine 47(17) e4-e4

WGO 2009 *Practice Guidelines: World Gastroenterology Organisation practice guideline: Probiotics and prebiotics.*

CHAPTER 2 - BENEFICIAL PROPERTIES OF LACTIC ACID BACTERIA NATURALLY PRESENT IN DAIRY PRODUCTION ENVIRONMENT

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Title page

Beneficial properties of lactic acid bacteria naturally present in dairy production environment

Running title: Beneficial properties of LAB from dairy environment

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Abstract

Lactic acid bacteria isolated from dairy environment were evaluated for beneficial potential. Preliminary screening and PCR analysis were applied to select and identified through 16s rRNA sequencing 15 LAB strains: *Lactobacillus* (n = 11; *Lb. casei* MSI1, *Lb. casei* MSI5, *Lb. casei* MRUV1, *Lb. casei* MRUV6, *Lb. acidophilus* MVA3, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSIV2, *Lb. harbinensis* MSI3, *Lb. plantarum* MLE5, *Lb. plantarum* MSI2 and *Lb. fermentum* SIVGL1), *Pediococcus* (n = 2; *P. acidilactici* MSI7 and *P. pentosaceus* MLEV8) and *Weissella* (n = 2; *W. paramesenteroides* MRUV3 and *W. paramesenteroides* MSAV5). All strains showed resistance to low pH and to presence of bile salt concentrations. API ZYM test characterized enzymatic activity between strains and high β-galactosidase activity was observed in 13 strains. All strains presented resistance after 3 hours on simulated gastric and 4 hours on intestinal conditions, ability to auto and co-aggregate with *L. monocytogenes* Scott A, *Enterococcus faecalis* ATCC 19443 and *Lactobacillus sakei* ATCC 15521 and high cell surface hydrophobicity. Most of the strains were positive for *map* and *EFTu* beneficial genes. All strains showed strong bile salts deconjugation and lactose assimilation. All 15 strains can have beneficial potential for contributing to the health-related functional properties.

Keywords: beneficial bacteria isolation, beneficial bacteria characterization, lactic acid bacteria, human health.

Introduction

Probiotics are defined as living organisms that benefit consumer health when ingested in adequate concentration (Guidelines 2009). The current trend of probiotic products consumption is due to the fact that consumers are increasingly looking for life quality. Health and well-being are directly linked to good nutrition associated with physical activity and lifestyle (Ouwehand and Röytiö 2014). Thus, probiotic strains would be inserted in this good nutrition concept in order to assist with health maintenance, through the prevention, control and treatment of diseases (Joint 2001).

There is a need to invest in research to isolate and characterize beneficial bacteria with probiotic potential in order to meet this demand by consumers. Dairy production environment are important sources of beneficial strains and fermented products are still the main sources of probiotic bacteria (Karimi et al. 2012). Based on this, lactic acid bacteria (LAB) are recognised for their probiotic properties in addition to being considered important with regard to dairy products and nutrition (Ashraf and Shah 2014).

Thus, the aim of the study was to explore the dairy production environment as a source of LAB strains with probiotic potential.

Materials and Methods

Screening

Samples and microbiological analysis

Raw milk, swab from Holstein cows and goat saliva and vaginal mucosa, ruminal boluses, consumption water, and silage were collected from Universidade Federal de Viçosa, Minas Gerais state, Brazil. Five samples were collected, totalling 10 samples, and they were kept refrigerated and subjected to laboratory analysis.

Lactic acid bacteria isolation and characterisation

All samples were subjected to ten-fold dilution using 0.85% NaCl (w/v). Selected dilutions were pour-plated in Man, Rogosa and Sharpe (MRS, Oxoid Ltd., Basingstoke, England) agar and MRS supplemented with 10 mg/L vancomycin (Sigma Aldrich, St. Louis, MO, USA) according to Colombo et al. (2014), both incubated at 37°C for 48 h in aerobic conditions. After incubation, colonies were enumerated and randomly

selected representative colonies were selected (10% of the observed count) and subjected to Gram staining and catalase reaction testing. Preliminary LAB characterised isolates (Gram positive and catalase-negative) were freeze-dried and stored at –20°C. The isolates were subjected to further microbiological analysis, as described in the following sections.

Gastric pH resistance

Gastric pH resistance was performed according to Argyri et al. (2013). Bacterial cells from overnight cultures (MRS, 37°C, 18 h) were centrifuged (10,000 x g for 5 min), and washed twice with phosphate-buffered saline (PBS, pH 7.2), before being re-suspended in PBS solution, adjusted with HCl to pH 2.0, 2.5 and 3.0. Resistance was assessed in three repetitions and duplicates in terms of viable colony counts on MRS agar after incubation at 37°C for 0 and 3 h, reflecting the time spent by food in the stomach. The resistance to low pH was performed according to Todorov et al. (2011) with some modifications. The isolates were grown at 37°C in MRS broth adjusted to pH 2.0, 2.5 and 3.0 with HCl and the culture was until 3 x 10^7 CFU/mL. All tests were conducted in sterile flat-bottom 96-well microtitre plates (NUNC, Thermo Scientific, Waltham, MA, EUA). In order to compare the count with the absorbance reading, optical density (OD) readings were recorded at 650 nm at zero time and after three hours of incubation at 37°C and aerobic condition, using a microtitreplate reader (Bio Tek Instruments Inc., Winooski, VT, USA). Cultures grown in MRS broth corrected to pH 7.2 served as control. Experiments were performed in three repetitions and duplicates.

Bile resistance

Bacterial cells from overnight cultures (MRS, 37°C, 18 h) were centrifuged (10,000 x g for 5 min), and washed twice with PBS (pH 7.2), before being re-suspended in PBS solution (pH 8.0), containing 0.5% (w/v) and 3% (w/v) bile salts (Sigma). Resistance was assessed in three repetitions and duplicates in terms of viable colony counts on MRS agar (Oxoid) after incubation at 37°C for 0 and 4 h, reflecting the time spent by food in the small intestine (Argyri et al. 2013). In addition, the resistance to bile salts was performed according to Todorov et al. (2011) with some modifications. The isolates were grown at 37°C in MRS broth containing 0.5% (w/v) and 3% (w/v) of bile salts (Sigma). All tests were conducted in sterile flat-bottom 96-well microtitre plates (NUNC, Thermo Scientific). In order to compare the count with the absorbance reading, OD readings were recorded at 650 nm at zero time and after four hours of incubation at

37°C, aerobiosis, using a microtitreplate reader (Bio Tek Instruments Inc., Winooski, VT, USA). Cultures grown in MRS broth without bile served as control. Experiments were performed in three repetitions and duplicates.

Molecular identification

Based on previous results, 82 from 500 isolates were selected. DNA was isolated according to the manufacturer's protocol using a ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). DNA concentrations were determined using NanoDrop (Thermo Scientific). Rep-PCR was performed according the protocol described by Dal Bello et al. (2010) using a single primer as stated in Table 1. PCR products were subject to electrophoresis on 2% (w/v) agarose gels for 6 h at a constant voltage of 75 V, in 0.5x Tris/Borate/EDTA buffer (TBE). Gels were stained using GelRed (Biotium Inc., Hayward, CA, USA), and recorded using a transilluminator LPIX (Loccus Biotecnologia, São Paulo, SP, Brazil). Further differentiation of LAB strains was also performed by random amplification of polymorphic DNA (RAPD-PCR) performed according to Todorov et al. (2010) with primers OPL-01, OPL-02, OPL-04, OPL-05, OPL-14 and OPL-20 (Table 1) (Kit L of the RAPD lomer kits, Operon Biotechnologies, Cologne, Germany). The amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels, stained with GelRed (Biotium Inc., Hayward, CA, USA) and 0.5× TBE buffer at 100 V for 2 h and visualized under UV light. Taxonomic identification was confirmed by sequencing of PCR-amplified 16S rRNA using the universal pair of primers 8F and 1512R (Felske et al. 1997). Sequencing of the amplicones was done in at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of São Paulo (São Paulo, SP, Brazil). Obtained sequences were compared to known sequences in GenBank using the Basic Local Alignment Search Tool (BLAST).

Detection of enzymatic activity

The 15 LAB selected were submitted to detection of enzymatic activity by the API ZYM Kit (bioMérieux, Basingstoke, Hants) according to the manufacture manual. The following enzymes were tested: alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valinearylamidase, cysteinearylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Table 1. PCR primers and conditions used for the detection of genes implicated in beneficial properties in lactic acid bacteria strains.

Target	Sequence gene	Function	References
Rep-PCR	GTGGTGGTGGTGGTG	Differentiation	Dal Bello *et al.* (2010)
RAPD PCR	OPL-01: GGCATGACCT	Differentiation	Todorov *et al.* (2010)
	OPL-02: TGGGCGTCAA		
	OPL-04: GACTGCACAC		
	OPL-05: ACGCAGGCAC		
	OPL-14: GTGACAGGCT		
	OPL-20: TGGTGGACCA		
16S rRNA	8F: CACGGATCCAGACTTTGATYMTGGCTCAG	Sequencing	Felske *et al.* (1997)
	1512R: GTGAAGCTTACGGYTAGCTTGTTACGACTT		
EF1249	F: GCGGTCGACAAACGAGGGATTTATTATG	Fibrinogen binding protein	Fortina *et al.* (2008)
	R: CTGGCGGCCGCGTTTAATACAATTAGGAAGCAGA		
EF2380	F: GCGGTCGACGACATCTATGAAAACAAT	Membrane-associated zinc metalloprotease	Fortina *et al.* (2008)
	R: TCCGCGCCGCCTTAAACTTTCTCCTT		
EF2662	F: GGCGTCGACCACTTAAACTGATAGAGAGGAAT	Choline binding protein	Fortina *et al.* (2008)
	R: CGCGCCGCAATTAATTATTAACTAGTTTCC		
prgB	F: GCCGTCGACTCGAGGAGAATGATACATGAAT	Surface protein	Fortina *et al.* (2008)
	R: CCTGCGGCCGCGTCCTTCTTTTCGTCTTCAA		
EFTu	F: TTCTGGTCGTATCGATCGTG	Adhesion-like factor	Ramiah *et al.* (2007)
	R: CCACGTAATAACGCACCAAC		
map	F: TGGATTCTGCTTGAGGTAAG	Mucus adhesion genes	Ramiah *et al.* (2007)
	R: GACTAGTAATAACGCGACCG		
mub	F: GTAGTTACTCAGTGACGATCAATG	Mucus adhesion genes	Ramiah *et al.* (2007)
	R: TAATTGTAAAGGTATAATCGGAGG		

Beneficial properties

Resistance to simulated gastric and intestinal human conditions

The resistance of the selected strains to gastric and intestinal conditions was evaluated through an *in vitro* model according to dos Santos et al. (2015). Overnight cultures of each strain were used to inoculate MRS broth (Oxoid) at 2× 10^8 CFU/mL, and an aliquot of 1 mL was serially diluted in peptone water, pour plated onto acidified MRS agar (pH 5.4), and incubated aerobically at 37 °C for 72 h to determine the bacterial concentrations (CFU/mL) at time 0. To simulate gastric conditions, 6 mL of the cell suspension was diluted in 10 mL of an artificial gastric fluid consisting of a sterile electrolyte solution (6.2 g/LNaCl, 2.2 g/LKCl, 0.22 g/L $CaCl_2$, and 1.2 g/L $NaHCO_3$, pH 2.5) supplemented with 0.3% pepsin (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 h at 37 °C under continuous agitation (150 rpm; Dubnoff Bath, Tecnal, Piracicaba, SP, Brazil). A one-millilitre aliquot was removed to determine the CFU/mL, as described previously. To simulate the passage through the small intestine, 2 mL of the remaining suspension was diluted in 8 mL artificial duodenal secretion (pH 7.2) consisting of 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5% bile salts (Oxgall, Merck, Darmstadt, Germany), and 0.1% pancreatin (Sigma-Aldrich). After 3 h of incubation at 37°C under continuous agitation (150 rpm), 1 mL aliquots were removed for determination of the final CFU/mL. The assay was performed three times for each strain, and the cultures enumeration was done in duplicate. The survival rate (SR) of strains after gastric and enteric simulation were calculated according to (Wang et al. 2009), using the equation: SR (%) = [log CFU N/log CFU N_0] × 100, where N_0 and N are the population values before and after the assay. Mean counts of log populations were compared by ANOVA and Tukey ($p < 0.05$) using XLSTAT 2016.01.26192 (AddinSoft, New York, NY, USA).

Aggregation, co-aggregation and hydrophobicity properties

Aggregation abilities of the 15 selected LAB were tested by the method previously proposed by Todorov et al. (2011) and Zhang et al. (2011). Briefly, cells from overnight cultures of the investigated LAB (10^8 CFU/mL) were centrifuged (6,000 × g for 10 min), washed three times with PBS (pH 7.3), re-suspended in the same buffer and homogenised. Concentration of the suspensions were standardised to 0.3 OD at 660 nm. One millilitre of cell suspension was transferred to a 2 mL sterile plastic cuvette and the OD at 660 nm was recorded using a spectrophotometer (Ultraspec 2000). Cells

suspensions were incubated at 37 °C for 1 h without agitation. For the determination of OD_{60}, the cultures were centrifuged at 300 g for 2 min and OD determined for the obtained supernatants. Auto-aggregation was determined using the following equation: % auto-aggregation = $[(OD_0 - OD_{60}) / OD_0] * 100$. OD_0 refers to the initial OD, and OD_{60} refers to the OD determined after 60 min.

For evaluation of co-aggregation, the 15 selected strains were grown in 10 mL of MRS and *L. monocytogenes* Scott A, *Enterococcus faecalis* ATCC 19443 and *Lactobacillus sakei* ATCC 15521 in BHI and MRS, at 37 °C (Todorov et al. 2011). Cells were centrifuged after 24 h (6000 x g for 10 min), washed, resuspended and diluted in PBS (pH 7.3), to OD 0.3 at 660 nm. One millilitre of each cell suspension was transferred to a 2 mL sterile plastic cuvette and the OD at 660 nm was recorded over 60 min using a spectrophotometer (Ultraspec 2000). The degree of co-aggregation was determined by OD readings of paired studied culture and co-aggregation combined suspensions (in ratio 500 µl and 500 µl of each suspension). Cells were centrifuged (300 x g for 2 min) and the OD at 660 nm of the supernatant was determined. Co-aggregation was calculated using the following equation: % co-aggregation = $[(OD_0 - OD_{60}) / OD_{60}] \times 100$. OD_0 refers to the initial OD, taken immediately after the relevant strains were paired. OD_{60} refers to the OD of the supernatant after 60 min. Experiments were conducted in three repetitions and duplicates on two separate occasions.

The test for bacterial adhesion to hydrocarbons was carried out according to Doyle and Rosenberg (1995). The 15 selected LAB were grown in MRS broth at 37 °C for 18 h. Cells were centrifuged (6,700 x g for 6 min), washed twice with quarter-strength Ringer's solution (sodium chloride, potassium chloride, calcium chloride and sodium bicarbonate), re-suspended in the same solution and the OD at 580 nm was determined. A sample of 1.5 mL cell suspension was added to 1.5 mL of n-hexadecane (Sigma) and vortexed for 2 min. The aqueous and organic phases were allowed to separate for 30 min at room temperature. One millilitre of the aqueous phase was removed and the OD at 580 nm was determined. The experiment was repeated and the average optical density value determined. The percentage hydrophobicity was calculated as follows: % hydrophobicity = $[(OD_{580}$ reading 1 – OD_{580} reading 2) / OD_{580} reading 1] x 100. Experiments were conducted in three repetitions and duplicates.

Finally, DNA obtained from the selected strains was subject for a PCR analysis for presence of genes (Table 1) related to the adhesion characteristics. The target genes used were *EF2380, EF2662, prgB, EF1249* (Fortina et al. 2008), *map, mub* and *EFTu* (Ramiah et al. 2007)

Bile salt deconjugation

To evaluate the strains ability to perform bile salt deconjugation, overnight cultures of each isolate were streaked on MRS agar plates previously prepared containing 0.5% (w/v) of the sodium salts of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC), and glycodeoxycholic acid (GDC) (Sigma-Aldrich). After aerobic incubation at 37 °C for 72 h, the presence of an opaque halo around colonies was considered positive for bile salt deconjugation (dos Santos et al. 2015). The test was performed in two independent experiments in duplicate.

β-galactosidase activity

The β-galactosidase activity of 15 selected strains was assessed employing sterile filter paper discs impregnated with o-nitrophenyl-β-D-galactopyranose (ONPG Discs, Fluka, Buchs, Switzerland), according to the manufacturer instructions. Overnight culture of each strain was streaked on MRS agar plates and incubated aerobically at 37 °C for 48 h. A colony of each strain was picked up and emulsified in a tube containing ONPG disc added with 0.1 mL of sterile 0.85% (w/v) sodium chloride solution. The tubes were incubated at 37 °C and observed at an interval of 1 h, for up to 6 h. The release of a yellow chromogenic compound, o-nitrophenol, indicates a positive result for the production of β-galactosidase. The test was performed in two independent experiments in duplicate.

Lactose assimilation

The ability of 15 LAB strains to metabolize lactose was tested by strains cultivation in modified MRS with 2% lactose as single carbon source for 24 h at 37 °C. Cultures obtained on same conditions but on MRS with 2% glucose as carbon source were used as control. Strains growth was estimated by viable cell counts after plating 10-fold serial dilutions on MRS agar medium (Pelinescu et al. 2011). Mean counts of log populations were compared by ANOVA ($p < 0.05$) using XLSTAT 2016.01.26192 (AddinSoft).

Results and Discussion

Screening

Based on the preliminary screening test for pH and bile resistance of the isolated LAB, from 500 isolates, 15 were selected for further analysis. From 500 isolates that were selected from the initial tests on survival pH and bile 394 were both Gram positive and

catalase negative. The last stage before conducting proper tests for beneficial activity was survival in extreme conditions within the gastrointestinal tract; results were considered positive for growth in MRS broth with low pH and high concentration of bile salts. After these screening tests, from 394 isolates, 82 were able to resist in pH 2.0 and 3 % of bile (in MRS broth) and were selected and submitted to molecular fingerprinting as described previously. Results showed that from the 82 tested strains, 15 can be considered unique, so were selected for taxonomical identification by sequencing of the PCR-amplified 16S rRNA. *Lb. casei* MSI1 and MSI5, *Lb. acidophilus* MVA3, *Lb. harbinensis* MSI3, *Lb. plantarum* MLE5 and MSI2 and *P. acidilactici* MSI7 were isolated using MRS, and *Lb. casei* MRUV1 and MRUV6, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSIV2, *Lb. fermentum* SIVGL1, *P. pentosaceus* MLEV8, *W. paramesenteroides* MRUV3 and MSAV5 were isolated using MRS-V. MRS-V medium have good potential to be applied in isolation of LAB with beneficial potential. The presence of vancomycin is important in order to inhibit the several other bacteria in the screening process. Colombo et al. (2014) previously applied this medium in isolation and selection of LAB from different origin. The intrinsically vancomycin resistant of some species, MRS-V becomes an option to select probiotic cultures that have this characteristic as they have specific cellular wall to this characteristic of resistance to this antibiotic (Nelson 1999). We have isolated eight LAB strains from silage (*Lb. casei* MSI1 and MSI5, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSI3 and MSIV2, *Lb. fermentum* SIVGL1, *Lb. plantarum* MSI2 and *P. acidilactici* MSI7), three from cow rumen (*Lb. casei* MRUV1 and MRUV6 and *W. paramesenteroides* MRUV3), two strains from cow milk (*Lb. plantarum* MLE5 and *P. pentosaceus* MLEV8), one strain from cow vaginal mucosa (*Lb. acidophilus* MVA3) and one from cow oral mucosa (*W. paramesenteroides* MSAV5). Previous studies demonstrated the presence of LAB with probiotic potential in the dairy environment (Banwo et al. 2013; Otero et al. 2006). From our knowledge, this is the first report of isolating *Lb. casei* and *W. paramesenteroides* from cow rumen.

Resistance to gastric pH and high bile concentration that is released in gut are key features for cultures to be able to resist the unfavourable conditions of gastrointestinal tract. As shown in Figures 1 and 2, the 15 selected LAB strains have a high survival rate in performed treatments. Although the results show similarity in low pH survival, it is noteworthy that this resistance is strain-specific. These results are in agreement with the findings of Vinderola and Reinheimer (2003) as regards the greater resistance of probiotic bacteria to low pH than other LAB. García-Ruiz et al. (2014) have reported

that *Lactobacillus* and *Pediococcus* strains were capable of surviving at low pH values. To the best of our knowledge, we are the first to investigate this for pH and bile resistance for *W. paramesenteroides.*

Figure 1 shows that tested strains were able to survive in gastric pH. None of the tested cultures presented a population decrease higher than 1 log. Similar results can be observed for changes in OD (Figure 1). *Lb. casei* MSI5 and MRUV6, *Lb. acidophilus* MVA3, *Lb. harbinensis* MSI3 and MSIV2, *Lb. fermentum* SIVGL1, *Lb. plantarum* MSI2, *P. acidilactici* MSI7 and *W. paramesenteroides* MSAV5 were the cultures that presented higher survival rates when compared to other strains. Bile salts at different concentrations affected the survival of tested strains. Among the 15 LAB strains selected for their good resistance to low pH, all strains exhibited fairly good bile tolerance after four hours of incubation in presence of bile salts (Figure 2). Similar results were also observed for changes in OD (Figure 2). The strains that exhibit higher sensitivity to treatment with bile salts were *Lb. casei* MSI1 and MRUV1, *Lb. acidophilus* MVA3 and *W. paramesenteroides* MSAV5. Moreover, all 15 tested LAB strains are able to resist to bile concentrations in intestine. Results obtained for bile salts are in agreement with the findings of Vinderola and Reinheimer (2003).

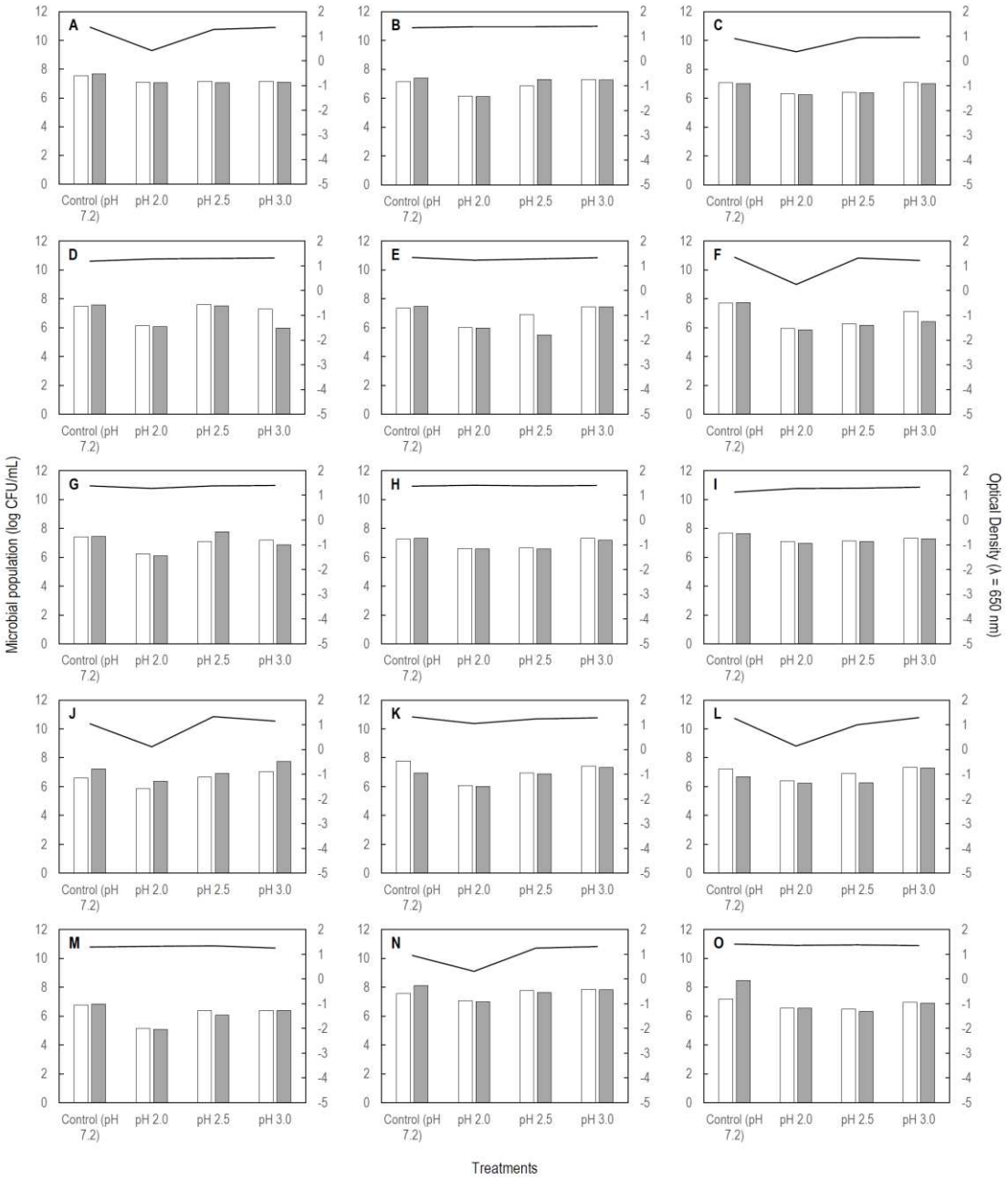

Figure 1. Resistance of LAB to effect of low pH as determined at 0h and 3h in non-growing conditions (results are expressed as $\log_{10}$ CFU/mL) and growth of LAB for 18h after been exposed to the effect of low pH for 3h (results are expressed as OD 650 nm determined on microplate reader). A: *Lactobacillus casei* MSI1; B: *Lb. casei* MSI5; C: *Lb. casei* MRUV1; D: *Lb. casei* MRUV6; E: *Lb. acidophilus* MVA3; F: *Lb. nagelli* MSIV4; G: *Lb. harbinensis* MSI3; H: *Lb. harbinensis* MSIV2; I: *Lb. fermentum* SIVGL1; J: *Lb. plantarum* MLE5; K: *Lb. plantarum* MSI2; L: *Pediococcus pentosaceus* MLEV8; M: *P. acidilactici* MSI7; N: *Weissella paramesenteroides* MRUV3; O: *W. paramesenteroides* MSAV5. The white bars represent the counts of the LAB strains at the initial time (zero) and the grey bars represent the count after 3 h incubated in the different pH treatments. The solid line represents the values of optical density in the different pH treatments.

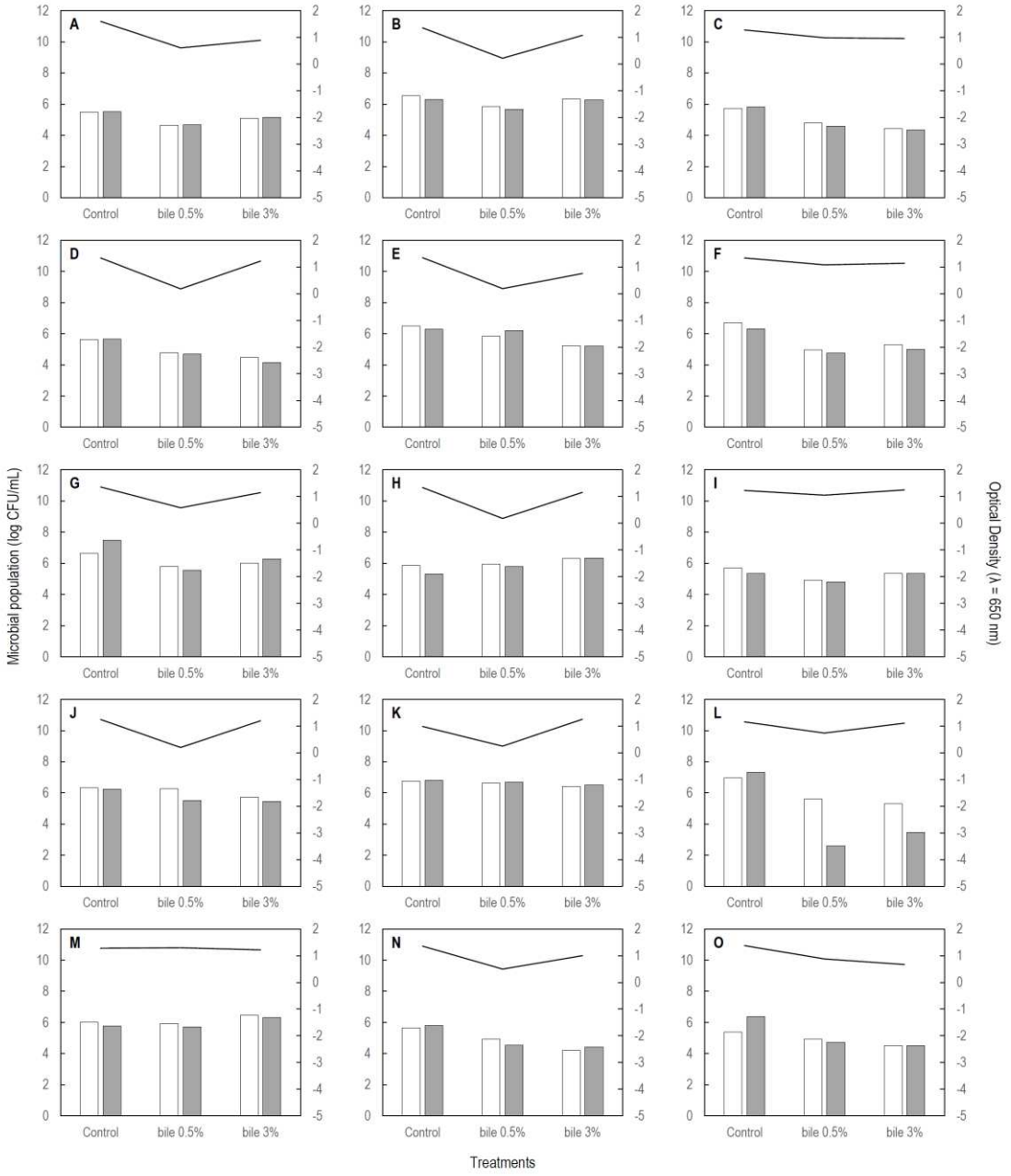

Figure 2. Resistance of LAB to effect of bile salts as determined at 0h and 4h in non-growing conditions (results are expressed as $\log_{10}$ CFU/mL) and growth of LAB for 18h after been exposed to the effect of bile salts for 4h (results are expressed as OD 650 nm determined on microplate reader). A: *Lactobacillus casei* MSI1; B: *Lb. casei* MSI5; C: *Lb. casei* MRUV1; D: *Lb. casei* MRUV6; E: *Lb. acidophilus* MVA3; F: *Lb. nagelli* MSIV4; G: *Lb. harbinensis* MSI3; H: *Lb. harbinensis* MSIV2; I: *Lb. fermentum* SIVGL1; J: *Lb. plantarum* MLE5; K: *Lb. plantarum* MSI2; L: *Pediococcus pentosaceus* MLEV8; M: *P. acidilactici* MSI7; N: *Weissella paramesenteroides* MRUV3; O: *W. paramesenteroides* MSAV5. The white bars represent the counts of the LAB strains at the initial time (zero) and the grey bars represent the count after 4 h incubated in the different bile treatments. The solid line represents the values of optical density in the different bile treatments.

The enzymatic activity patterns recorded for the tested strains by using API ZYM are presented in Table 2. All tested strains presented positive results for leucine arylamidase, acid phosphatase and naphtholphosphohydrolase. *Lb. harbinensis* MSIV2 generated positive results for production of 17 enzymes part of API ZYM kit and negative for α-mannosidase and α-fucosidase. Lipase, trypsin and β-glucuronidase activities were absent in most of strains and α-mannosidase and α-fucosidase activities were absent in all 15 tested strains. In general, the enzymatic profiles of our *Lactobacillus* strains are similar to those reported by other authors (Georgieva et al. 2009; Pisano et al. 2014). The enzymatic activity is important for many functions of the tested culture. For example, strains with high peptidase but with low proteinase and esterase-lipase activities may be useful in improving body and texture in cheese production and reducing bitterness (Georgieva et al. 2009). β-galactosidase activity is useful in improving lactose tolerance in the gut and it is very important for a probiotic culture (de Vrese et al. 2001). Our results show the production of this enzyme for 13 of the 15 LAB strains. Arora et al. (1990) compared the enzymatic profile of 20 *Lb. casei* strains and indicated the presence of proteinase, peptidase and esterase-lipase activities. The strong peptidase and esterase activities in *Lactobacilli* has been highlighted by the great importance in cheese production, like accelerated maturation and enzyme modification. Therefore, these results are very important for both industrial and research purposes. Tzanetakis and Litopoulou-Tzanetaki (1989) examined *P. pentosaceus* strains with API ZYM system: leucine and valine aminopeptidase were found in all strains and β-galactosidase, esterase, esterase-lipase and acid phosphatase were detected in most of the strains, in agreement the results recorded for *P. pentosaceus* MLEV8 (Table 2). However, *N*-acetyl-β-glucosamidase, β-glucosidase, lipase, cysteine were negative for this strain, unlike the results observed by Tzanetakis and Litopoulou-Tzanetaki (1989). To the best of our knowledge, we are the first reporting on API ZYM system on *W. paramesenteroides.* This way, we can evaluate other tests for characterisation the probiotic potential of these 15 LAB in a more accurate way, as follows.

Table 2. Enzymatic profile of the studied 15 lactic acid bacteria (LAB) strains determined by APIZYM test.

LAB		Alkaline phosphatase	Esterase	Esterase lipase	Lipase	Leucine arilamidase	Valine arilamidase	Cistine arilamidase	Trypsin	α- chymotrypsin	Acid phosphatase	Naphthol phosfohydrolase	α- galactosidase	β- galactosidase	β- glucuronidase	α- glucosidase	β- glucosidase	N-acetyl-β-glucosaminidase	α- manosidase	α- fucosidase
Lb. casei	MSI1	-	+	+	-	+	+	-	-	-	+	+	+	+	-	+	+	-	-	-
	MSI5	+	+	+	-	+	+	+	-	+	+	+	-	+	-	+	+	+	-	-
	MRUV1	-	-	-	+	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-
	MRUV6	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-
Lb. acidophilus	MVA3	-	+	+	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-
Lb. nagelli	MSIV4	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-
Lb. harbinensis	MSI3	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	-
	MSIV2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Lb. fermentum	SIVGL1	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-
Lb. plantarum	MLE5	+	+	+	-	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-
	MSI2	-	+	+	-	+	+	+	-	-	+	+	-	+	-	+	+	+	-	-
P. pentosaceus	MLEV8	-	+	+	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-
P. acidilactici	MSI7	-	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	+	-	-
W. paramesenteroides	MRUV3	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-
	MSAV5	-	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-

Beneficial properties

For a better simulation of the *in vitro* gastrointestinal environment, tests were also performed for gastric and intestinal phase causing the passage through adversities found in this environment are closer to actual possible. The results are shown in Table 3. The tested strains were able to survive and even multiply in gastric phase reaching survival rates (SR) values higher than 91%. In the intestinal phase, most cultures decreased their populations, reaching values between 46 and 102%. However, all of the tested LAB cultures were able to survive the gastrointestinal phase. Pisano et al. (2014) recorded survival rates with more than 98% for *Lactobacillus* strains. The same results are shown by Caggia et al. (2015) and dos Santos et al. (2015). Vidhyasagar and Jeevaratnam (2013) showed that *Pediococcus* strains are able to survive both gastric and intestinal phase. We did not find results for *W. paramesenteroides* strains in the literature.

As mentioned in this work, in addition to surviving the gastrointestinal host environment, probiotic bacteria must adhere to the gastrointestinal tract if beneficial properties are related to the colonisation of host by probiotic LAB. The auto-aggregation ability allows bacteria to persist in intestinal mucosa and thus promote their beneficial effects to the host. LAB co-aggregation is also considered a positive point since these same strains are able to manifest effects against pathogens. Auto-aggregation and co-aggregation results are shown in Table 4. The auto-aggregation as the co-aggregation showed to be strain specific. This was previously observed for other strains with beneficial properties (Todorov et al. 2011). *Lb. plantarum* MLE5 and *P. pentosaceus* MLEV8 showed the highest auto-aggregation properties, with 91.7% and 96.3%, respectively. All 15 tested LAB strains showed results with more than 50% auto-aggregation and 14 of them with more than 60%. Fifteen strains showed 50% co-aggregation with *Listeria monocytogenes* Scott A. The results for co-aggregation with *E. faecalis* ATCC 19443 showed that 11 strains exhibited more than 50%, while 4 strains presented between 40 and 50%. Co-aggregation with *Lb. sakei* ATCC 15521, which is non-pathogenic, may play an important role in facilitating the presence of this species in the human GI tract. *Lb. casei* MRUV1 did not show good results of co-aggregation, presenting results of 33.7%. The other 13 strains showed results between 48 and 63%. Thus, all 15 LAB strains showed co-aggregation abilities with pathogens tested but the degree of co-aggregation was variable like specific strain. Our results are in agreement with dos Santos et al. (2015), that also find a large range for auto-aggregation in *Lactobacillus* (28.8 – 87.7 %) and co-aggregation with *Lb. monocytogenes* up to 60%. Caggia et al. (2015) also found large range for auto-

aggregation in *Lactobacillus* (5 to 68%). Todorov et al. (2011) reported that *Lactobacillus* presented low levels of co-aggregation with pathogens (*L. monocytogenes* and *E. faecalis*) and high levels with *Lb. sakei*. Lee et al. (2014) showed that for *Pediococcus* strains possessed strong auto-aggregation phenotypes ranging between 65 and 69%. The authors also showed that *Pediococcus* had 24 to 29% co-aggregation, and *Lactobacillus* presented 16 to 26% of co-aggregation with *E. faecalis* ATCC 29212. Vidhyasagar and Jeevaratnam (2013) showed that a *Pediococcus* strain exhibited maximum aggregation of 89%, which reveals clumping of cells and that this strain effectively aggregated with *L. monocytogenes* with a range of 81%. Anandharaj et al. (2015) reported that a *Weissella* strain showed maximum auto-aggregation (79%) and co-aggregation (68%) with *Escherichia coli* MTCC 1089.

Cell surface hydrophobicity is the capacity of bacteria to present interactions with mucosal cells. All tested strains showed high hydrophobicity (96 to 100%, Table 4). The average hydrophobicity values registered for investigated 15 LAB strains are shown in Table 4. Caggia et al. (2015) showed good hydrophobicity to cell surface for *Lactobacillus* strain, with a percentage higher than 70%. Vidhyasagar and Jeevaratnam (2013) showed high hydrophobicity for *Pedicoccus* strains (55 to 79%). Todorov et al. (2011) recorded high levels of hydrophobicity for *Lactobacillus* and *Pediococcus* strains (43 to 79% and 51.3%, respectively). However, we did not find any studies for *W. paramesenteroides* related to the hydrophobicity. The presence of the main surface proteins genes can be associated to high adhesion ability, competitive exclusion of pathogens and adhesion-dependent stimulation of the immune system by probiotic LAB strains (Ramiah et al. 2007). The results for the presence of genes associated to adhesion properties are summarized in Table 4. The genes *EF1249, EF2380* and *prgB* were not identified in any of the tested isolates, while *EFTu* was identified in 13 strains, *map* in nine strains, *EF2662* in six strains, and *mub* in four strains. *EF2662, map, mub* and *EFTu* play a key role in the action of probiotic cultures mechanisms: *EF2662* is a novel putative binding protein gene and it is responsible for recognizing adhesive matrix molecules facilitating the adhesion (Solheim et al. 2011). *Map* and *mub* are mucus adhesion genes and this makes it possible to adhesion in GI tract mucosal cells. *EFTu* is an adhesion-like factor gene that also aids in cell adhesion. *map, mub* and *EFTu* are up-regulated in the presence of mucus, proportional to increasing concentrations (Ramiah et al. 2007).

Table 3. Survival of selected 15 lactic acid bacteria (LAB) strains to *in vitro* gastrointestinal conditions (gastric and intestinal phases).

LAB	Identification	Population (log CFU/mL)*			Survival rate** (SR%)	
		Control (Initial)	Gastric phase	Intestinal phase	Gastric phase	Intestinal phase
Lb. casei	MSI1	6.54±0.00	6.79±0.01	5.76±1.40	103.8	88.1
	MSI5	8.74±0.00	8.79±0.00	6.76±2.13	100.6	77.4
	MRUV1	9.15±0.00^{a}	8.80±0.00^{a}	7.30±0.40^{b}	96.3	79.8
	MRUV6	8.29±0.00^{c}	8.79±0.01^{a}	8.41±0.02^{b}	106.0	101.4
Lb. acidophilus	MVA3	7.71±0.00^{c}	7.84±0.00^{b}	8.00±0.01^{a}	101.7	71.0
Lb. nagelli	MSIV4	8.95±0.00^{a}	8.71±0.01^{b}	7.90±0.03^{c}	97.3	88.3
Lb. harbinensis	MSI3	8.87±0.00^{a}	8.77±0.01^{a}	5.46±2.20^{b}	98.9	61.6
	MSIV2	7.98±0.00^{a}	7.84±0.00^{b}	4.04±0.08^{c}	98.2	50.6
Lb. fermentum	SIVGL1	8.53±0.00^{a}	7.77±0.01^{b}	4.17±0.06^{c}	91.1	48.9
Lb. plantarum	MLE5	8.48±0.00^{a}	7.77±0.00^{ab}	6.00±1.41^{b}	91.6	70.8
	MSI2	7.78±0.00^{b}	8.78±0.00^{a}	3.98±0.09^{c}	112.9	51.2
P. pentosaceus	MLEV8	8.26±0.00^{a}	7.84±0.00^{ab}	7.31±0.56^{b}	94.9	88.5
P. acidilactici	MSI7	9.00±0.00^{a}	8.79±0.00^{b}	4.20±0.08^{c}	97.7	46.7
W. paramesenteroides	MRUV3	6.78±0.00^{b}	6.79±0.00^{b}	7.98±0.03^{a}	100.1	117.7
	MSAV5	7.39±0.00	6.79±0.01	5.25±1.52	91.9	70.8

*Average values ± standard deviations, three independent repetitions; values followed by different letters are significantly different by ANOVA and Tukey ($p < 0.05$);
**SR(%) = [log CFU N/ log CFU N_0] x 100, where N0 and N are the population values before and after the assay, respectively.

Table 4. Autoaggregation of lactic acid bacteria (LAB) and coaggregation rates between LAB and *L. monocytogenes* Scott A, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521 (%), cell hydrophobicity, lactose assimilation, presence of genes associated to beneficial properties tested in 15 selected LAB.

LAB	Identification	Autoaggregation (%)	Coaggregation rates (%)			Hydrophobicity (%)	Beneficial related genes						
			L. monocytogenes Scott A	*E. faecalis* ATCC 19443	*L. sakei* ATCC 15521		*EF1249*	*EF2380*	*EF2662*	*prgB*	*EFTu*	*map*	*mub*
Lb. casei	MSI1	68.9	61.6	53.5	55.9	99.8	-	-	-	-	+	+	-
	MSI5	73.6	71.5	49.1	56.6	99.9	-	-	+	-	+	-	-
	MRUV1	70.0	54.0	52.3	33.7	98.6	-	-	+	-	+	+	-
	MRUV6	68.7	58.9	52.4	56.8	99.1	-	-	+	-	+	-	+
Lb. acidophilus	MVA3	69.4	69.6	55.8	59.9	99.4	-	-	+	-	+	+	-
Lb. nagelli	MSIV4	63.4	67.4	60.6	65.3	99.3	-	-	-	-	+	-	+
Lb. harbinensis	MSI3	78.7	64.0	52.6	65.6	99.6	-	-	-	-	+	-	-
	MSIV2	71.1	64.3	66.4	48.3	98.8	-	-	+	-	+	+	+
Lb. fermentum	SIVGL1	62.9	65.5	49.7	55.8	97.3	-	-	-	-	-	-	-
Lb. plantarum	MLE5	91.7	62.8	57.1	64.1	97.2	-	-	-	-	+	+	-
	MSI2	86.9	57.8	50.9	57.4	99.5	-	-	-	-	+	-	+
P. pentosaceus	MLEV8	96.3	72.7	49.7	62.4	97.1	-	-	-	-	-	+	-
P. acidilactici	MSI7	78.9	60.4	59.6	63.0	98.4	-	-	+	-	+	+	-
W. paramesenteroides	MRUV3	50.0	57.9	46.2	58.0	98.8	-	-	-	-	+	+	-
	MSAV5	67.4	62.1	55.0	63.1	96.2	-	-	-	-	+	+	-

*** Coaggregation test in plates: *Weissella paramesenteroides* with *E. faecalis*: 13 mm of inhibition halo (the only culture).

LAB bacteria that present deconjugate capacity are desired for use in probiotic products for human consumption because studies show the capacity reduction of serum cholesterol by these cultures (Begley et al. 2006). The obtained results shown that all 15 investigated LAB strains recorded high ability to grow on MRS agar plates containing 0.5% (w/v) sodium salts of TC, TDC, GC and GDC (data not shown). Our results are in agreement with dos Santos et al. (2015), Caggia et al. (2015) and Anandharaj et al. (2015), who also recorded strong deconjugation efficiency for *Lactobacillus, Pediococcus* and *Weissella* strains.

On ONPG dics (Fluka), only five LAB strains showed strong β-galactosidase activity, among them *Lb. casei* MSI1 and MRUV6, *Lb. plantarum* MLE5, *Lb. fermentum* SIVGL1 and *Lb. nagelli* MSIV4 (data not shown). Between them, *Lb. nagelli* MSIV4 was negative in API ZYM Kit (bioMérieux) as mentioned before and also in this Kit, 13 strains had strong β-galactosidase activity. The API ZYM Kit (bioMérieux) is more sensitive than other tests and for this reason, more cultures were positive in it than other test of β-galactosidase activity. In the same purpose could be used to lactose intolerance, the 15 LAB strains were tested for the ability to assimilate lactose, and all these strains were able to assimilate lactose. Besides that, eight LAB strains showed better assimilation with lactose than glucose being they: *Lb. casei* MSI5, MRUV1 and MRUV6, *Lb. acidophilus* MVA3, *Lb. harbinensis* MSIV2, *P. pentosaceus* MLEV8 and *W. paramesenteroides* MRUV3 and MSAV5. These results shown the good ability to produce β-galactosidase enzyme or to assimilate lactose and they are therefore able to alleviate lactose intolerance. This is an important point for the dairy industry, because these cultures will be able to grow in milk based environment. Also, these bacteria should provide sensorial properties to fermented products. Our results are in agreement with those found by Lee et al. (2014) and Vidhyasagar and Jeevaratnam (2013).

Conclusion

There is significant increase in the role of probiotics exists in nutrition and human medicine. However, specific researches regarding isolation, characterization, safety and application of these microorganisms in food are still necessary, as are accurate studies on their mechanisms of action to promote the desired benefits. We identified the dairy environment as a potential source of LAB strains possessing beneficial features, and the obtained 15 LAB presented promising characteristics for use as probiotic cultures.

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References

Anandharaj M, Sivasankari B, Santhanakaruppu R, Manimaran M, Rani RP, Sivakumar S (2015) Determining the probiotic potential of cholesterol-reducing *Lactobacillus* and *Weissella* strains isolated from gherkins (fermented cucumber) and south Indian fermented koozh Res Microbiol 166:428-439 doi:http://dx.doi.org/10.1016/j.resmic.2015.03.002

Argyri AA, Zoumpopoulou G, Karatzas KG, Tsakalidou E, Nychas GE, Panagou EZ, Tassou CC (2013) Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests Food Microbiol 33:282-291 doi:http://dx.doi.org/10.1016/j.fm.2012.10.005

Arora G, Lee BH, Lamoureux M (1990) Characterization of Enzyme Profiles of *Lactobacillus casei* Species by a Rapid API ZYM System Journal of dairy science 73:264-273 doi:http://dx.doi.org/10.3168/jds.S0022-0302(90)78669-9

Ashraf R, Shah NP (2014) Immune system stimulation by probiotic microorganisms Crit Rev Food Sci Nutr 54:938-956

Banwo K, Sanni A, Tan H (2013) Technological properties and probiotic potential of *Enterococcus faecium* strains isolated from cow milk J Appl Microbiol 114:229-241 doi:10.1111/jam.12031

Begley M, Hill C, Gahan CGM (2006) Bile salt hydrolase activity in probiotics Applied and Environmental Microbiology 72:1729-1738

Caggia C, De Angelis M, Pitino I, Pino A, Randazzo CL (2015) Probiotic features of *Lactobacillus* strains isolated from Ragusano and Pecorino Siciliano cheeses Food Microbiol 50:109-117 doi:http://dx.doi.org/10.1016/j.fm.2015.03.010

Colombo M, Oliveira AEZ, Carvalho AF, Nero LA (2014) Development of an alternative culture medium for the selective enumeration of *Lactobacillus casei* in fermented milk Food Microbiol 39:89-95 doi:http://dx.doi.org/10.1016/j.fm.2013.11.008

Dal Bello B, Rantsiou K, Bellio A, Zeppa G, Ambrosoli R, Civera T, Cocolin L (2010) Microbial ecology of artisanal products from North West of Italy and antimicrobial activity of the autochthonous populations LWT - Food Sci Technol 43:1151-1159

de Vrese M, Stegelmann A, Richter B, Fenselau S, Laue C, Schrezenmeir J (2001) Probiotics - compensation for lactase insufficiency The American Journal of Clinical Nutrition 73:421S-429S

dos Santos KMO et al. (2015) Artisanal Coalho cheeses as source of beneficial *Lactobacillus plantarum* and *Lactobacillus rhamnosus* strains Dairy Science & Technology 95:209-230

Doyle RJ, Rosenberg M (1995) Measurement of microbial adhesion to hydrophobic substrata Methods in enzymology 253:542

Felske A, Rheims H, Wolterink A, Stackebrandt E, Akkermans ADL (1997) Ribosome analysis reveals prominent activity of an uncultured member of the class *Actinobacteria* in grassland soils Microbiology 143:2983-2989 doi:doi:10.1099/00221287-143-9-2983

Fortina MG et al. (2008) A survey on biotechnological potential and safety of the novel *Enterococcus* species of dairy origin, *E. italicus* Int J Food Microbiol 123:204-211 doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2008.01.014

García-Ruiz A, González de Llano D, Esteban-Fernández A, Requena T, Bartolomé B, Moreno-Arribas MV (2014) Assessment of probiotic properties in lactic acid bacteria isolated from wine Food Microbiol 44:220-225 doi:http://dx.doi.org/10.1016/j.fm.2014.06.015

Georgieva R, Iliev I, Haertlé T, Chobert J, Ivanova I, Danova S (2009) Technological properties of candidate probiotic *Lactobacillus plantarum* strains International Dairy Journal 19:696-702 doi:http://dx.doi.org/10.1016/j.idairyj.2009.06.006

Guidelines WP (2009) World Gastroenterology Organisation practice guideline: Probiotics and prebiotics vol 10. Arabian Journal of Gastroenterology, vol 1.

Joint (2001) FAO/WHO Expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Córdoba, Argentina

Karimi R, Mortazavian AM, Amiri-Rigi A (2012) Selective enumeration of probiotic microorganisms in cheese Food Microbiology 29:1-9 doi:10.1016/j.fm.2011.08.008

Lee KW, Park JY, Sa HD, Jeong JH, Jin DE, Heo HJ, Kim JH (2014) Probiotic properties of *Pediococcus strains* isolated from jeotgals, salted and fermented Korean sea-food Anaerobe 28:199-206 doi:http://dx.doi.org/10.1016/j.anaerobe.2014.06.013

Nelson RRS (1999) Intrinsically vancomycin-resistant Gram-positive organisms: clinical relevance and implications for infection control Journal of Hospital Infection 42:275-282 doi:10.1053/jhin.1998.0605

Otero MC, Morelli L, Nader-Macías ME (2006) Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle Letter of Applied Microbiology 43:91-97 doi:10.1111/j.1472-765X.2006.01914.x

Ouwehand AC, Röytiö H (2014) Probiotic fermented foods and health promotion. Advances in Fermented Foods and Beverages: Improving Quality, Technologies and Health Benefits. Elsevier, 1st Edition, 586p.

Pelinescu D et al. (2011) Selection and characterization of the probiotic potential of some lactic acid bacteria isolated from infant feces. Romanian Biotechnological Letters 16:6178-6189

Pisano MB et al. (2014) Preliminary evaluation of probiotic properties of *Lactobacillus* strains isolated from sardinian dairy products. BioMed Research International 2014:9 doi:10.1155/2014/286390

Ramiah K, van Reenen CA, Dicks LMT (2007) Expression of the mucus adhesion genes *Mub* and *MapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423, monitored with real-time PCR. International Journal of Food Microbiology 116:405-409 doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2007.02.011

Solheim M, Brekke MC, Snipen LG, Willems RJL, Nes IF, Brede DA (2011) Comparative genomic analysis reveals significant enrichment of mobile genetic elements and genes encoding surface structure-proteins in hospital-associated clonal complex 2 *Enterococcus faecalis* BMC Microbiol 11:3

Todorov SD, Furtado DN, Saad SMI, Tome E, Franco BDGM (2011) Potential beneficial properties of bacteriocin-producing lactic acid bacteria isolated from smoked salmon J Appl Microbiol 110:971-986

Todorov SD et al. (2010) Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium* Food Microbiology 27:869-879 doi:http://dx.doi.org/10.1016/j.fm.2010.05.001

Tzanetakis N, Litopoulou-Tzanetaki E (1989) Biochemical activities of *Pediococcus pentosaceus* isolates of dairy origin Journal of Dairy Science 72:859-863 doi:http://dx.doi.org/10.3168/jds.S0022-0302(89)79178-5

Vidhyasagar V, Jeevaratnam K (2013) Evaluation of *Pediococcus pentosaceus* strains isolated from Idly batter for probiotic properties in vitro Journal of Functional Foods 5:235-243 doi:http://dx.doi.org/10.1016/j.jff.2012.10.012

Vinderola CG, Reinheimer JA (2003) Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance Food Research International 36:895-904 doi:http://dx.doi.org/10.1016/S0963-9969(03)00098-X

Wang AN, Yi XW, Yu HF, Dong B, Qiao SY (2009) Free radical scavenging activity of *Lactobacillus fermentum* in vitro and its antioxidative effect on growing–finishing pigs J Appl Microbiol 107:1140-1148

Zhang Y et al. (2011) Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food Microbiol Res 167:27-31 doi:http://dx.doi.org/10.1016/j.micres.2011.02.006

CHAPTER 3 - VIRULENCE POTENTIAL AND ANTIMICROBIAL RESISTANCE OF BENEFICIAL LACTIC ACID BACTERIA ISOLATED FROM DAIRY PRODUCTION ENVIRONMENT

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Virulence potential and antimicrobial resistance of lactic acid bacteria with benefic potential isolated from dairy systems

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Abstract

In a previous work, 15 lactic acid bacteria strains were isolated from a dairy environment, identified as *Lactobacillus* (n = 11), *Pediococcus* (n = 2) and *Weissella* (n = 2) and selected due to their beneficial potential. The present study aimed to assess their virulence potential and antimicrobial resistance, in order to evaluate for safety usage by the food industry. The production of virulence factors (hemolysis, gelatinase, lipase, deoxyribonuclease and biogenic amines: lysine, tyrosine, histidine and ornithine) was assessed by phenotypic methods at 25 °C and 37 °C, as well as the resistance to 17 antimicrobials. The isolates were also subjected to PCR to identify the presence of 49 genes associated with virulence factors. None of the strains presented hemolytic activity or the production of gelatinase, lipase, deoxyribonuclease and tested biogenic amines. Of the 15 selected cultures, for 12 types of antibiotics in the disc diffusion method, all strains were resistant for oxacillin and sulfa/trimethoprim, 14 were resistant to gentamicin, 11 were resistant to clindamycin, nine strains were resistant to vancomycin, eight strains to rifampicin, five were resistant to erythromycin, four were resistant to tetracycline, two strains were resistant to ampicillin, one strain was resistant to chloramphenicol and none was resistant for imipenem. For a quantitative test of the antibiogram, five antibiotics were selected. All 15 strains were resistant to vancomycin, two for rifampicin, one for gentamicin and one for chloramphenicol. Regarding the virulence related genes, 19 genes from 49 tested were present in some strains. Results showed that five cultures showed the presence of the *int* gene, four cultures showed the presence of the *ant(4')-Ia* gene, three cultures were positive for *vanC2*, *cpd* and *tdc*, two cultures for *vanA, tet(K), tet(S), ermA, bcrR, mur-2ed, asa1* and *ccf*, and one culture was positive for *vanC1, ermB, aph(3')-IIIa, aac(6')-le-aph(2")-Ia, bcrB* and *hyl.* Based on the obtained results, the isolates presented safe behaviors, leading to further studies to assess their potential usage as beneficial cultures in the food industry.

Keywords: beneficial bacteria, virulence potential, antibiotic resistance, safety use of lactic acid bacteria.

1. Introduction

Lactic acid bacteria (LAB) has a long and known history about its use in fermented products: the fermentation process as in the production of antimicrobial substances including lactic acid and other organic acids and bacteriocins (Holzapfel et al., 1995; Leroy and de Vuyst, 2004). In addition to the mentioned benefits, LAB can also be characterized as potentially probiotic according to the beneficial effects on consumer health and safety use (EFSA, 2005; Kechagia et al., 2013). Among LAB, *Lactobacillus* species are usually described as possessing beneficial properties, as well as some *Streptococcus, Leuconostoc, Pediococcus* and *Enterococcus* (Fontana et al., 2013; Holzapfel et al., 2001). In addition, non-LAB bacteria are also widely described as probiotics, being used in food products for human and animal consumption; among these organisms, *Bifidobacterium* and *Propionibacterium* are often described and used in fermented products (Aragon et al., 2010; Meile et al., 2008).

Increasingly studies have made significant advances in the selection and characterization of beneficial cultures, focusing on benefits to consumer health (Joint, 2001). Moreover, it is necessary to assess the safety of these cultures to be used for human consumption, as adverse effects may be present despite the benefits caused by them (Joint, 2006). Many pathogenic mechanisms used by bacteria cause disease in human hosts (Wilson et al., 2002). These pathogens express a wide variety of molecules that bind to host cells and thereby stimulate different responses. In addition to different pathogenicity strategies, several factors are reported as dangerous, with special concerns related to the presence of antimicrobial resistance genes (Bautista-Gallego et al., 2013; Zhang et al., 2009), gene exchange induction in the gastrointestinal tract (Reenen and Dicks, 2010; Salyers et al., 2004), indiscriminate use of antibiotics in human and veterinary medicine and animal breeding for several decades (Muñoz et al., 2014). These factors result in a significant risk to public health, for this reason, it is necessary to further studies of bacteria that are introduced into the food chain in order to avoid propagation resistance and cause virulence risk for humans and animals (Reenen and Dicks, 2010).

Culture considered probiotic, in addition to resist, persist in the gastrointestinal tract and provide benefits to the host, must present safety properties. Recognition as GRAS (Generally Recognized as Safe) is extremely important, and to be classified as such, the probiotic strain should present a minimal possibility of antimicrobial resistance transfer and should be safe for food use with proven health promoting effects (e.g., non-invasive

in epithelial cell line models, production of anti-inflammatory rather than proinflammatory cytokines) (Joint, 2006). However, reports from recent decades show that GRAS status is not enough to indicate that a strain is totally safe, once other virulence factors are not considered in the evaluation (Rubio et al., 2014; Sharma et al., 2014). Deep research for the safety of each specific strain needs to be performed in order to confirm the safety of the strain to be applied in the food fermentation processes or as a beneficial culture for human or animal consumption. In addition, for these beneficial cultures to be considered totally safe for human health, they cannot cause disease (such as bacteremia), they cannot have toxic or metabolic effects and should be able to transfer antibiotic resistance (Sharma et al., 2014; Snydman, 2008). Thus, the studies with bacteria with beneficial potential will require characterization of their virulence potential.

Furthermore, this study allowed us to characterize previously isolated LAB cultures as potential beneficial cultures and detect any virulence factors for safe use in food industry.

2. Material and Methods

2.1. Bacterial strains and growth conditions

In a previous study (Chapter 2 of this thesis), a culture collection composed of 15 isolates selected on preliminary screening from 500 isolates, obtained from dairy environment was characterized as beneficial potential LAB by phenotypical and molecular methods, then identified as *Lactobacillus* spp. (n = 11), *Pediococcus* spp. (n = 2) and *Weissella* spp. (n = 2) by sequencing and grouped according to rep-PCR and RAPD PCR profiles. Based on these characteristics, *Lb. casei* MSI1, *Lb. casei* MSI5, *Lb. casei* MRUV1, *Lb. casei*MRUV6, *Lb. acidophilus* MVA3, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSI3, *Lb. harbinensis* MSIV2, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5, *Lb. plantarum*MSI2, *P. pentosaceus* MLEV8, *P. acidilactici* MSI7, *W. paramesenteroides* MRUV3 and *W. paramesenteroides* MSAV5 were selected for the present study. The isolates were stored in de Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) supplemented with 25% (v/v) glycerol at −80 °C. For use, stock cultures were streaked on MRS agar (Oxoid), incubated at 37 °C for 24 h, and then isolated colonies were transferred to MRS broth (Oxoid) and incubated at 37 °C for 24 h.

2.2. Virulence activity

The selected 15 LAB strains were subjected to phenotypical tests of hemolytic activity, gelatinase production, lipase production and DNAse activity to identify their virulence activity, according to Barbosa et al. (2010). All experiments were performed at least in three repetitions and duplicates.

Hemolytic activity. Hemolytic activity was assessed by streaking the cultures onto trypticase soya agar (Oxoid) added to defibrinated horse blood at 5% (v/v), then incubated at 25 °C and 37 °C, for 24 h. The hemolysis formed by each isolate was classified as total or β-hemolysis (clear halos around the colonies), partial or α-hemolysis (greenish halos around the colonies), and absent or γ-hemolysis.

Gelatinase production. Gelatinase production was verified by spotting 1 µL aliquots of the examined cultures onto the surface of Luria Bertani agar (LB - Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA) added to gelatin (BD) at 3% (w/v), then incubated at 25 °C and 37 °C, for 48 h. After incubation, the plates were maintained at 4 °C for 4 h, and the hydrolysis of gelatin was recorded by the formation of opaque halos around the colonies.

Lipase production. Lipase production was assessed by spotting 1 µL of cultures onto LB (BD) added to $CaCl_2$ (Sigma-Aldrich, at 0.2%, w/v) and Tween (Sigma-Aldrich, at 1%, v/v), then incubated at 25 °C and 37 °C, for 48 h. The formation of transparent halos around the colonies was recorded as lipase production.

DNAse activity. DNAse was identified by spotting in 1 µL aliquots of the cultures on the surface of DNAse methyl green agar (BD), incubated at 25 °C and 37 °C, for 48 h. Positive results were identified by the formation of clear halos around the colonies.

2.3. Biogenic amines production

The production of biogenic amines was evaluated according to Bover-Cid and Holzapfel (1999). Decarboxylase production was induced by five consecutive transfers of 0.5 mL aliquots of the cultures in MRS broth (Oxoid) supplemented with pyridoxal-5-phosphate at 0.005 % (w/v, Sigma-Aldrich) and with each one of the biogenic amine precursors at 0.1% (w/v): lysine, tyrosine, ornithine, and histidine (Sigma Aldrich). Each culture was incubated at 25 °C and 37 °C, for 24 h, and the end cultures were streaked in duplicate onto decarboxylase agar, an MRS agar modified according to

Joosten and Northolt (1989), supplemented with one of each biogenic amine precursor described as above, at 1 % (w/v). The plates were incubated at 25 °C and 37 °C for 4 days, and positive results were recorded when the color changed from yellow to purple.

2.4. Antibiotic resistance

Cultures of the 15 selected isolates were subjected to phenotypical analysis of antibiotic resistance Disks Oxoid® (Unipath Ltd, Basingstoke, UK) and Etest® strips (bioMérieux SA, Marcy l'Etoile, France). The cultures were diluted using NaCl 0.85% (w/v) until turbidity similar to 0.5 McFarland scale was reached. Cultures were then homogeneously swabbed onto the surface of MRS (Oxoid) plates. After this, the discs of antibiotics were placed onto the plated surface, and the plates were incubated at 37 °C for 24 h. The following antibiotics were used: oxacillin (1μg/disk), sulpha/trimethoprim (25 μg/disk), tetracycline (30 μg/disk), imipenem (10 μg/disk), ampicillin (10 μg/disk), erythromycin (15 μg/disk), vancomycin (30 μg/disk), rifampicin (5 μg/disk), gentamicin (10 μg/disk), penicillin (10 U/disk), clindamycin (2 μg/disk) and chloramphenicol (30 μg/disk). Halo (inhibition zone) formation around the discs was measured and classified as presenting resistance (R), intermediate resistance (IR), or sensitivity (S) (according to the instructions from the manufacturer and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014). After this, the minimum inhibitory concentrations (MIC) of five antibiotics encompassing nearly all important classes were determined: vancomycin, gentamicin, chloramphenicol, ampicillin and rifampicin. Considering the halo formation around the strips, the MIC was estimated (μg/mL) for each antibiotic against each strain, which was classified as presenting resistance (R) or sensitivity (S), according to the instructions of the manufacturer for rifampicin, and the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016) for the other antibiotics tested. We considered the breakpoint values suggested by Clinical and Laboratory Standards Institute (CLSI, 2014) for *Streptococcus* spp.

2.5. Detection of virulence and resistance genes

DNA from the isolates was extracted using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) and DNA concentration was estimated using NanoDrop2000 (Thermo Scientific Inc., Waltham, MA, USA). The presence of 49

virulence, antibiotic resistance and biogenic amine related genes was investigated: *vanA, vanB, vanC1, vanC2* and *vanC2/C3* (vancomycin resistance); *tet(K), tet(L), tet(M), tet(O)* and *tet(S)* (tetracycline resistance); *ermA, ermB* and *ermC* (erytromycin resistance); *catA* (chloramphenicol resistance); *aph(2'')-lb, ant(4')-la, aph(2'')-ld, aph(2'')-lc* and *aph(3')-llla* (aminoglycoside antibiotics family resistance); *aac(6')-le-aph(2'')-Ia* (gentamycin and aminoglycoside resistance); *vat(E)* (streptogramin resistance); *bcrB, bcrD* and *bcrR* (bacitracin resistance); *ant(6)-la* (streptomycin resistance); *mur-2ed* (specific for *E. durans*); *aac(6')-li* (specific for *E, faecium*); *mur-2* (specific for *E. hirae*), $Ddl_{E.\ faecalis}$ (specific for *E. faecalis*); *ace* (adhesion of collagen of *E. faecalis*); *asa1* (aggregation substance); *cyt2* (cytolisin and hemolytic endotoxins); *esp* (enterococcal surface protein); *efaA* (endocarditis antigen); *cob, cpd* and *ccf* (chemotactic for human leukocytes andfacilitated conjugation); *sprE, fsrA, fsrB, fsrC* and *gelE* (gelatinase production); *odc* (ornithinedecarboxylase); *tdc* (tyrosinedecarboxylase); *hdc1* and *hdc2* (histidinedecarboxylase); *hyl* (hyaluronidase); *int* and *int-Tn* (transposon integrase gene). The primers and references for PCR conditions are described in Table 1. PCR products were separated on agarose gels from 0.8 to 2.0% (w/v) in 0.5× TBE and stained with TAE buffer containing 0.5 μg/mL gel red (Sigma-Aldrich, Inc., St. Louis, MO, USA).

Table 1. PCR primers and conditions used for the detection of genes implicated in antibiotic resistance and virulence in LAB strains

Target gene	Sequence gene	References
vanA	F: TCTGCAATAGAGATAGCCGC R: GGAGTAGCTATCCCAGCATT	Martin-Platero et al. (2009)
vanB	F: GCTCCGCAGCCTGCATGGACA R: ACGATGCCGCCATCCTCCTGC	Paulsen et al. (2003)
vanC1	F: GCTGAAATATGAAGTAATGACCA R: CGGCATGGTGTTGATTTCGTT	Miele et al. (1995)
vanC-1	F: GGTATCAAGGAAACCTC R: CTTCCGCCATCATAGCT	Dutka-Malen et al. (1995)
vanC2	F: CTCCTACGATTCTCTTG R: CGAGCAAGACCTTTAAG	Dutka-Malen et al. (1995)
vanC2/C3	F: CTCCTACGATTCTCTTG R: CGAGCAAGACCTTTAAG	Dutka-Malen et al. (1995)
tet(K)	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACTCATTCCAGAAGCA	Aarestrup et al. (2000)
tet(L)	F: CATTTGGTCTTATTGGATCG R: ATTACACTTCCGATTTCGG	Aarestrup et al. (2000)
tet(M)	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	Aarestrup et al. (2000)
tet(O)	F: GATGGCATACAGGCACAGAC R: CAATATCACCAGAGCAGGCT	Aarestrup et al. (2000)
tet(S)	F: TGGAACGCCAGAGAGGTATT R: ACATAGACAAGCCGTTGACC	Aarestrup et al. (2000)

ermA	F: TCTAAAAAGCATGTAAAAGAA R:CTTCGATAGTTTATTAATATTAG	Sutcliffe et al. (1996)
ermB	F: CATTTAACGACGAAACTGGC R: GGAACATCTGTGGTATGGCG	Jensen et al. (1999)
ermC	F: ATCTTTGAAATCGGCTCAGG R: CAAACCCGTATTCCACGATT	Jensen et al. (1999)
catA	F: GGATATGAAATTTATCCCTC R: CAATCATCTACCCTATGAAT	Aarestrup et al. (2000)
aph(2'')-Ib	F: TATGGATCCATGGTTAACTTGGACGCTGAGAT R:TAAGCTTCCTGCTAAAATATAAACATCTCTGCT	Kao et al. (2000)
ant(4')-Ia	F: CAAACTGCTAAATCGGTAGAAGCC R: GGAAAGTTGACCAGACATTACGAACT	Vakulenko and Mobashery (2003)
aph(2'')-Id	F: GTGGTTTTTACAGGAATGCCATC R: CCCTCTTCATACCAATCCATATAACC	Fortina et al. (2008)
aph(2'')-Ic	F:CCACAATGATAATGACTCAGTTCCC R: CCACAGCTTCCGATAGCAAGAG	Fortina et al. (2008)
aph(3')-llla	F: GCCGATGTGGATTGCGAAAA R: GCTTGATCCCCAGTAAGTCA	Fortina et al. (2008)
aac(6')-Ie-aph(2'')-Ia	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	Van de Klundert and Vliegenthart (1993)
vat(E)	F: ACGTTACCCATCACTATG R: GCTCCGATAATGGCACCGAC	Duh et al. (2001)
bcrB	F: AAAGAAACCGACTGCTGATA R: GCTTACTTGTATAGCAGAGA	Manson et al. (2004)
bcrD	F: AGGATTCGGCCGAATGGCACTTGATTTTAT R: GTTTCTTCGCGAAATTGCCGTTATAAGTAA	Manson et al. (2004)
bcrR	F: AACAAACAGGGAGCGGCCGCATGGAATTTA R: TGATGTTCGCGATTTCATTCCCATCTGCTT	Manson et al. (2004)
ant(6)-Ia	F: ACTGGCTTAATCAATTTGGG R: GCCTTTCCGCCACCTCACCG	Fortina et al. (2008)
mur-2$_{ed}$	F: AACAGCTTACTTGACTGGACGC R: GTATTGGCGCTACTACCCGTATC	Robredo et al. (1999)
aac(6')-Ii	F: GCGGTAGCAGCGGTAGACCAAG R: GCATTTGGTAAGACACCTACG	Costa et al. (1993)
mur-2	F: CGTCAGTACCCTTCTTTTGCAGAGTC R: GCATTATTACCAGTGTTAGTGGTTG	Chu et al. (1992)
Ddl$_{E. faecalis}$	F: ATCAAGTACAGTTAGTCT R: ACGATTCAAAGCTAACTG	Dutka-Malen et al. (1995)
ace	F: GAATTGAGCAAAAGTTCAATCG R: GTCTGTCTTTTCACTTGTTTC	Martin-Platero et al. (2009)
asa1	F: GCACGCTATTACGAACTATGA R: TAAGAAAGAACATCACCACGA	Vankerckhoven et al. (2004)
cyt2	F: ACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTT	Vankerckhoven et al. (2004)
esp	F: AGATTTCATCTTTGATTCTTG R: AATTGATTCTTTAGCATCTGG	Vankerckhoven et al. (2004)
efaA	F: GCCAATTGGGACAGACCCTC R: CGCCTTCTGTTCCTTCTTTGGC	Martin-Platero et al. (2009)
cob	F: AACATTCAGCAAACAAAGC R: TTGTCATAAAGAGTGGTCAT	Eaton and Gasson (2001a)
cpd	F: TGGTGGGTTATTTTTCAATTC R: TACGGCTCTGGCTTACTA	Eaton and Gasson (2001a)
ccf	F: GGGAATTGAGTAGTGAAGAAG R: AGCCGCTAAAATCGGTAAAAT	Eaton and Gasson (2001a)
sprE	F: TTGAGCTCCGTTCCTGCCGAAAGTCATTC R: TTGGTACCGATTGGGGAACCAGATTGACC	Nakayama et al. (2002)

fsrA	F: ATGAGTGAACAAATGGCTATTTA R: CTAAGTAAGAAATAGTGCCTTGA	Nakayama et al. (2002)
fsrB	F: GGGAGCTCTGGACAAAGTATTATCTAACCG R: TTGGTACCCACACCATCACTGACTTTTGC	Nakayama et al. (2002)
fsrC	F: ATGATTTTGTCGTTATTAGCTACT R: CATCGTTAACAACTTTTTTACTG	Nakayama et al. (2002)
gelE	F: TATGACAATGCTTTTTGGGAT R: AGATGCACCCGAAATAATATA	Vankerckhoven et al. (2004)
odc	F: GTNTTYAAYGCNGAYAARCANTAYTTYGT R: ATNGARTTNAGTTCRCAYTTYTCNGG	Rivas et al. (2005)
tdc	F: GAYATNATNGGNATNGGNYTNGAYCARG R: CCRTARTCNGGNATAGCRAARTCNGTRTG	Rivas et al. (2005)
hdc1	F: AGATGGTATTGTTTCTTATG R: AGACCATACACCATAACCTT	Favaro et al. (2014)
hdc2	F: AAYTCNTTYGAYTTYGARAARGARG R: ATNGGNGANCCDATCATYTTRTGNCC	Favaro et al. (2014)
hyl	F: ACAGAAGAGCTGCAGGAAATG R: GACTGACGTCCAAGTTTCCAA	Vankerckhoven et al. (2004)
int-Tn	F: TGACACTCTGCCAGCTTTAC R: CCATAGGAACTTGACGTTCG	Barbeyrac et al. (1996)
int	F: GCGTGATTGTATCTCACT R: GACGCTCCTGTTGCTTCT	Gevers et al. (2003)

3. Results and Discussion

No investigated strains presented any virulence factors for hemolytic activity, gelatinase production, lipase production or deoxyribonuclease activity in *in vitro* tests at either 25 °C or 37 °C (data not shown). The same was verified for the *in vitro* detection of biogenic amine production. All the strains showed negative results for lysine, tyrosine, histidine and ornithine biogenic amines, as expected for safety cultures (Boyle et al., 2006; Vankerckhoven et al., 2008). The absence of hemolytic activity and biogenic amine production in *Lactobacillus* is in agreement with findings reported by Pisano et al. (2014). The production of extracellular enzymes and hemolytic activity were not exhibited by *Pediococcus* as shown by Borges et al. (2013). We did not find studies about biogenic amine production in *Pediococcus.* To the best of our knowledge, there is no study about hemolytic activity or extracellular enzymes for *W. paramesenteroides.* The two *W. paramesenteroides* strains investigated in our study did not shown positive results for the tests cited above, in contrast to the findings reported by Jeong and Lee (2015): they found that *W. paramesenteroides* was positive for two biogenic amines (histamine and tyramine).

Table 2 presents the results for antibiotic resistance tests: the disc diffusion method and Etest® strips (bioMérieux), one being qualitative and the other quantitative. The results

showed that most of the cultures were sensitive to most of the antibiotics disks tested. Regarding the screening test for antimicrobial resistance using the disc diffusion method, all strains were resistant for oxacillin and sulfa/trimethoprim, and just one (*Lb. harbinensis* MSIV2) was sensitive to gentamicin (all other 14 cultures were resistant). Most test cultures were also resistant to clindamycin (11 strains of 15), vancomycin (9 of 15) and rifampicin (8 of 15). No culture was resistant to more than seven of the 12 antibiotic types tested. *Lb. acidophilus* MVA3, *Lb. plantarum* MSI2 and *W. paramesenteroides* MRUV3 were the three strains that have more variable resistance to the tested antibiotics; they showed resistance to seven antibiotics. *Lb. casei* MSI1 and MRUV1, *Lb. harbinensis* MSI3, *Lb. plantarum* MLE5, *Lb. pentosaceus* MLEV8 and *W. paramesenteroides* MSAV5 showed resistance to six of 12 tested antibiotics. *Lb. casei* MSI5, *Lb. nagelli* MSIV4, *Lb. fermentum* SIVGL1 and *P. acidilactici* MSI7 showed resistance for five tested antibiotics. Moreover, *Lb. casei* MRUV6 and *Lb. harbinensis* MSIV2 were resistant to just four antibiotics. The results obtained in this study agree with those obtained by other authors (dos Santos et al., 2015; Han et al., 2015; Liu et al., 2009; Muñoz et al., 2014). The antimicrobial resistance profiles obtained by MIC are presented in Table 2. *Lb. harbinensis* MSI3 and *W. paramesenteroides* MRUV3 were the two strains that showed most results of resistance for the five tested antibiotics: they were sensitive just for rifampicin and gentamicin, (Table 2). Ampicillin and vancomycin were the two antibiotics that most strains showed resistance: 10 strains were resistant to ampicillin and 14 to vancomycin. Gentamicin, chloramphenicol and rifampicin were the antibiotics that showed most sensitive results for 15 LAB strains. The results obtained in this study agree with those obtained by other authors with regard to the susceptibility of the lactobacilli strains to the selected antibiotics (Danielsen and Wind, 2003; Korhonen et al., 2007; Muñoz et al., 2014; Rubio et al., 2014). In addition to the genus *Lactobacillus*, Munoz-Atienza et al. (2013) found antibiotic resistance in the *Weissella* and *Pediococcus* genera. These data corroborate the results obtained with respect to the 15 LAB strains antibiotic resistance profiles and suggest safety for use as a probiotic candidate. The presence of resistance to more than three antibiotics is not a problem in a medical setting, because they can also be highly sensitive to other relevant antibiotics (Muñoz et al., 2014).

Considering the tests for virulence related genes, *Lb. fermentum* SIVGL1 and *Lb. plantarum* MSI2 were the only two strains that presented negative results for all tested genes, and *Lb. harbinensis* MSI3 showed just one positive record (*asa1*). The *asa1* gene is responsible for aggregation substance capacity and this increases bacterial adherence

to renal tubular cells and heart endocardial cells, enhances internalization in intestinal epithelial cells, and has been shown to increase the valvular vegetation mass in an animal model of endocarditis (Vankerckhoven et al., 2004). *Lb. nagelli* MSIV4 (*int* and *ccf*), *Lb. harbinensis* MSIV2 (*vanC2* and *cpd*), *Lb. plantarum* MLE5 (*vanC1*and *aph(3')-IIIa*), *P. pentosaceus* MLEV8 (*aac(6')-Ie* and e*rmB*) and *P. acidilactici* MSI7 (*ermA*and *bcrR*) presented two virulence genes. *Lb. casei* MRUV1 (*vanA, ant(4')-Ia* and *int*) and MRUV6 (*ant(4')-Ia, tdc* and *crmA*), and *W. paramesenteroides* MRUV3 (*van*A*, ant(4')-Ia* and *int*) showed positive results for three genes. *Lb. casei* MSI1 (*mur-2ed, vanC2, tdc* and *bcrB*) and MSI5 (*vanC2, cpd, tetS* and *bcrB*), and *W. paramesenteroides* MSAV5 (*mur-2ed, cpd, int* and *hyl*) were positive for four genes. The strain that showed positive results for most genes investigated was *Lb. acidophilus* MVA3, which was positive for eight of them (*ant(4')-Ia, tetS, tdc, ermA, bcrB, tetK, ccf* and *asa1*). *VanC1,* v*anC2, vanA* (vancomycin), *ant(4´)-Ia* (aminoglycoside), *tet(S)* (tetracycline), *ermA* (erythromycin), *bcrR* (bacitracin), *tetK* (tetracycline), *aph(3')-IIIa* (aminoglycoside), *bcrB* (bacitracin), *aac(6')-Ie* (gentamycin and aminoglycoside), and *ermB* (erythromycin) are antibiotic resistance genes that are easily transferred to other microorganisms once they are located in conjugative plasmids (Eaton and Gasson, 2001b). *Mur-2ed* is a gene specific for *E. durans* (Robredo et al., 1999). *Cpd* and *ccf* are genes that facilitate conjugation (Eaton and Gasson, 2001a). *tdc* is tyrosine decarboxylase, *int* is a transposon integrase gene, *hyl* is hyaluronidase and *asa1* is aggregation substance (Gevers et al., 2003; Vankerckhoven et al., 2004). Many of these genes have not been reported in LAB strains and that is because many of them have been passed from one bacterium to another by gene transfer. None of the 15 tested strains were positive for the following genes: *vanB, vanC-1, vanC2/C3, tet(L), tet(M), tet(O), int-Tn, ermCcatA, aph(2'')-Ib, aph(2'')Id, aph(2'')-Ic, aph(3')-IIIa, vat(E), bcrD, ant(6)-la, mur-2, Ddl*$_{E.\ faecalis}$*, ace, cyt2, esp, efaA, cob, sprE, fsrA, fsrB, fsrC, gelE, odc, hdc1* and *hdc2*. The results obtained in this study agree with those obtained by other authors (dos Santos et al., 2015; Munoz-Atienza et al., 2013; Muñoz et al., 2014).

Table 2. Phenotypic and genotypic antibiotic resistance and resistance and virulence genes detected by PCR of lactic acid bacteria.

Specie	Strain	Phenotypic antibiotic resistance		Resistance and virulence gene (s) detected by PCR
		Discs of antibiotic	MIC ($\mu g.ml^{-1}$)	
Lb. casei	MSI1	OXA, GEN, ERY, CLI, SUL, RIF	VAN (AH), AMP (1.0)	*vanC2, bcrB, mur-2ed, tdc*
	MSI5	OXA, VAN, GEN, CLI, SUL	VAN (AH)	*vanC2, tet(S), bcrR, cpd*
	MRUV1	OXA, AMP, VAN, GEN, TET, SUL	VAN (AH), AMP (1.0)	*vanA, ant(4')-Ia, int*
	MRUV6	OXA, VAN, GEN, SUL	VAN (AH), AMP (1.0)	*ermA, ant(4')-Ia, tdc*
Lb. acidophilus	MVA3	OXA, VAN, GEN, ERY, CLI, SUL, RIF	VAN (AH), AMP (1.0), RIF (AH)	*tet(K), tet(S), ermA, ant(4')-Ia, bcrR, asa1, ccf, tdc*
Lb. nagelli	MSIV4	OXA, GEN, CLI, SUL, RIF	VAN (AH), AMP (50.0)	*ccf, int*
Lb. harbinensis	MSI3	OXA, VAN, GEN, TET, CLI, SUL	VAN (AH), AMP (1.0), GEN (AH), CHL (AH)	*asa1*
	MSIV2	OXA, VAN, CLI, SUL	VAN (AH), RIF (32.0)	*vanC2, cpd*
Lb. fermentum	SIVGL1	OXA, AMP, VAN, GEN, SUL	VAN (AH) , AMP (1.5)	
Lb. plantarum	MLE5	OXA, GEN, ERY, CLI, SUL, RIF	VAN (AH) , AMP (0.64)	*vanC1, aph(3')-IIIa*
	MSI2	OXA, VAN, GEN, CHL, CLI, SUL, RIF	VAN (AH)	
P. pentosaceus	MLEV8	OXA, GEN, ERY, CLI, SUL, RIF	VAN (AH), AMP (50.0)	*ermB, aac(6')-Ie-aph(2")-Ia*
P. acidilactici	MSI7	OXA, VAN, GEN, TET, SUL	VAN (AH)	*tet(K), int*
W. paramesenteroides	MRUV3	OXA, VAN, GEN, TET, CLI, SUL, RIF	VAN (AH), AMP (50.0), CHL (50.0), RIF (4.0)	*vanA, ant(4')-Ia, int*
	MSAV5	OXA, GEN, ERY, CLI, SUL, RIF		*mur-2ed, cpd, hyl, int*

VAN: vancomycin, GEN: gentamicin, CHL: chloramphenicol, RIF: rifampicin, OXA: oxacillin, AMP: ampicillin, ERY: erytromycin, CLI: clindamycin, SUL: Sulpha/Trimethoprim. AH: absence of inhibition zone. Breaking points have been determined according to manufacturer instructions (bioMérieux, France), and CLSI standard (CLSI, 2014).

4. Conclusion

Culture considered probiotic, in addition to resist, persist in the gastrointestinal tract and provide benefits to the host, must present safety properties. Benefic cultures have significant importance in nutrition and human medicine. However, specific studies regarding isolation, characterization, safety and application of these microorganisms in food are required, as accurate studies on the mechanism of action of these compounds in promoting the desired benefits are lacking. In addition, beneficial cultures characterized as such should be analyzed with respect to the potential virulence of the strains. The importance of this is because of virulence mechanisms also occur between beneficial bacteria by transfer or genetic mutation. Virulence studies promote knowledge about the isolated cultures to be safe for use in food industry. Almost all 15 selected LAB strains isolated from dairy environment should be regarded as safe to be used by the food industry because of the absence of acquired resistance determinants. As previously highlighted, the presence of resistance to more than three antibiotics is not a problem in a medical setting, because these compounds can also be highly sensitive to other relevant antibiotics. The 15 strains examined here could be potentially probiotic candidate microorganisms because of their capacity for survival in gastrointestinal tract environment and even promote beneficial effects to the host and also safety.

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References

Aarestrup, F.M., Agerso, Y., Gerner–Smidt, P., Madsen, M., Jensen, L.B., 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn Microbiol Infect Dis 37, 127-137.

Aragon, G., Graham, D.B., Borum, M., Doman, D.B., 2010. Probiotic therapy for Irritable Bowel Syndrome. Gastroenterology & Hepatology 6, 39-44.

Barbeyrac, B., Dupon, M., Rodriguez, P., Renaudin, H., Bébéar, C., 1996. A Tn1545-like transposon carries the tet (M) gene in tetracycline resistant strains of *Bacteroides ureolyticus*

as well as *Ureaplasma urealyticum* but not *Neisseria gonorrhoeae*. Journal of Antimicrobial Chemotherapy 37, 223-232.

Barbosa, J., Gibbs, P.A., Teixeira, P., 2010. Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. Food Control 21, 651-656.

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Research International 50, 135-142.

Borges, S., Barbosa, J., Silva, J., Teixeira, P., 2013. Evaluation of characteristics of Pediococcus spp. to be used as a vaginal probiotic. J Appl Microbiol 115, 527-538.

Bover-Cid, S., Holzapfel, W.H., 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int J Food Microbiol 53, 33-41.

Boyle, R.J., Robins-Browne, R.M., Tang, M.L.K., 2006. Probiotic use in clinical practice: what are the risks? The American Journal of Clinical Nutrition 83, 1256-1264.

Chu, C., Kariyama, R., Daneo-Moore, L., Shockman, G.D., 1992. Cloning and sequence analysis of the muramidase-2 gene from *Enterococcus hirae*. Journal of Bacteriology 174, 1619-1625.

CLSI, 2014. Performance standards for antimicrobial disc and dilution susceptibility tests for bacteria isolated from animals; approved standard. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA, p. 99.

Costa, Y., Galimand, M., Leclercq, R., Duval, J., Courvalin, P., 1993. Characterization of the chromosomal aac(6')-Ii gene specific for *Enterococcus faecium*. Antimicrob Agents Chemother 37, 1896-1903.

Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int J Food Microbiol 82, 1-11.

dos Santos, K.M.O., Vieira, A.D.S., Buriti, F.C.A., do Nascimento, J.C.F., de Melo, M.E.S., Bruno, L.M., Borges, M.F., Rocha, C.R.C., Lopes, A.C.S., Franco, B.D.G.M., 2015. Artisanal Coalho cheeses as source of beneficial *Lactobacillus plantarum* and *Lactobacillus rhamnosus* strains. Dairy Science & Technology 95, 209-230.

dos Santos, K.M.O., Vieira, A.D.S., Salles, H.O., Oliveira, J.S., Rocha, C.R.C., Borges, M.F., Bruno, L.M., Franco, B.D.G.M., Todorov, S.D., 2015. Safety, beneficial and technological properties of *Enterococcus faecium* isolated from Brazilian cheeses. Brazilian Journal of Microbiology 46, 237-249.

Duh, R., Singh, K.V., Malathum, K., Murray, B.E., 2001. In vitro activity of 19 antimicrobial agents against enterococci from healthy subjects and hospitalized patients and use of an ace gene probe from *Enterococcus faecalis* for species identification. Microbial Drug Resistance 7, 39-46.

Dutka-Malen, S., Evers, S., Courvalin, P., 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol 33, 24-27.

Eaton, T.J., Gasson, M.J., 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Applied and Environmental Microbiology 67, 1628-1635.

EFSA, E.F.S.A., 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, EFSA Panel on Additives and Products or Substances used in Animal Feed. EFSA Journal, p. 10.

EFSA, F.P., 2005. Opinion of the Scientific Panel on additives and products or substances used in animal feed (FEEDAP) on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance, EFSA Panel on Additives and Products or Substances used in Animal Feed.

EUCAST, 2016. European Committee on Antimicrobial Susceptibility Testing, in: EUCAST, V. (Ed.), Compliance of manufacturers of AST materials and devices with EUCAST guidelines.

FAO/WHO, 2001. FAO/WHO Expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, Córdoba, Argentina, pp. 1-4.

FAO/WHO, 2006. Health and nutritional properties and guidelines for evaluation. FAO/WHO Food and Nutrition, Rome Paper 85.

Favaro, L., Basaglia, M., Casella, S., Hue, I., Dousset, X., Franco, B.D.G.M., Todorov, S.D., 2014. Bacteriocinogenic potential and safety evaluation of non-starter *Enterococcus faecium* strains isolated from home made white brine cheese. Food Microbiol 38, 228-239.

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., Gil, A., 2013. Sources, isolation, characterisation and evaluation of probiotics. The British Journal of Nutrition 109 Suppl 2, S35-50.

Fortina, M.G., Ricci, G., Borgo, F., Manachini, P.L., Arends, K., Schiwon, K., Abajy, M.Y., Grohmann, E., 2008. A survey on biotechnological potential and safety of the novel *Enterococcus* species of dairy origin, *E. italicus*. Int J Food Microbiol 123, 204-211.

Gevers, D., Danielsen, M., Huys, G., Swings, J., 2003. Molecular characterization of tet (M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. Applied and Environmental Microbiology 69, 1270-1275.

Han, J., Chen, D., Li, S., Li, X., Zhou, W., Zhang, B., Jia, Y., 2015. Antibiotic susceptibility of potentially probiotic *Lactobacillus strains*. Italian Journal of Food Science 27, 8.

Holzapfel, W.H., Geisen, R., Schillinger, U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International Journal of Food Microbiology 24, 343-362.

Holzapfel, W.H., Haberer, P., Geisen, R., Björkroth, J., Schillinger, U., 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. The American Journal of Clinical Nutrition 73, 365s-373s.

Jensen, L.B., Frimodt-Moller, N., Aarestrup, F.M., 1999. Presence of *erm* gene classes in Gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiology Letters 170, 151-158.

Jeong, D., Lee, J., 2015. Antibiotic resistance, hemolysis and biogenic amine production assessments of *Leuconostoc* and *Weissella* isolates for kimchi starter development. LWT - Food Science and Technology 64, 1078-1084.

Joosten, H.M.L.J., Northolt, M.D., 1989. Detection, growth, and amine-producing capacity of lactobacilli in cheese. Applied and Environmental Microbiology 55, 2356-2359.

Kao, S.J., You, I.l., Clewell, D.B., Donabedian, S.M., Zervos, M.J., Petrin, J., Shaw, K.J., Chow, J.W., 2000. Detection of the high-level aminoglycoside resistance gene aph(2")-Ib in *Enterococcus faecium*. Antimicrob Agents Chemother 44, 2876-2879.

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., Fakiri, E.M., 2013. Health Benefits of Probiotics: A Review. ISRN Nutrition 2013, 7.

Korhonen, J.M., Sclivagnotis, Y., Wright, A.v., 2007. Characterization of dominant cultivable lactobacilli and their antibiotic resistance profiles from faecal samples of weaning piglets. J Appl Microbiol 103, 2496-2503.

Leroy, F., de Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science & Technology 15, 67-78.

Liu, C., Zhang, Z., Dong, K., Yuan, J.P., Guo, X.K., 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomedical and Environmental Sciences 22, 11.

Manson, J.M., Keis, S., Smith, J.M.B., Cook, G.M., 2004. Acquired bacitracin resistance in *Enterococcus faecalis* is mediated by an ABC transporter and a novel regulatory protein, BcrR. Antimicrob Agents Chemother 48, 3743-3748.

Martin-Platero, A.M., Valdivia, E., Maqueda, M., Martinez-Bueno, M., 2009. Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. International Journal of Food Microbiology 132, 24-32.

Meile, L., Le Blay, G., Thierry, A., 2008. Safety assessment of dairy microorganisms: *Propionibacterium* and *Bifidobacterium*. International Journal of Food Microbiology 126, 316-320.

Miele, A., Bandera, M., Goldstein, B.P., 1995. Use of primers selective for vancomycin resistance genes to determine van genotype in enterococci and to study gene organization in VanA isolates. Antimicrob Agents Chemother 39, 1772-1778.

Munoz-Atienza, E., Gomez-Sala, B., Araujo, C., Campanero, C., del Campo, R., Hernandez, P., Herranz, C., Cintas, L., 2013. Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. BMC Microbiol 13, 15.

Muñoz, M.C.C., Benomar, N., Lerma, L.L., Gálvez, A., Abriouel, H., 2014. Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. Int J Food Microbiol 172, 110-118.

Nakayama, J., Kariyama, R., Kumon, H., 2002. Description of a 23.9-kilobase chromosomal deletion containing a region encoding fsr genes which mainly determines the gelatinase-negative phenotype of clinical isolates of *Enterococcus faecalis* in urine. Applied and Environmental Microbiology 68, 3152-3155.

Paulsen, I.T., Banerjei, L., Myers, G.S.A., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D.E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science 299, 2071-2074.

Pisano, M.B., Viale, S., Conti, S., Fadda, M.E., Deplano, M., Melis, M.P., Deiana, M., Cosentino, S., 2014. Preliminary evaluation of probiotic properties of *Lactobacillus* strains isolated from sardinian dairy products. BioMed Research International 2014, 9.

Reenen, C.A., Dicks, L.M.T., 2010. Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: what are the possibilities? A review. Archives of Microbiology 193, 157-168.

Rivas, B., Marcobal, A., Muñoz, R., 2005. Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. FEMS Microbiology Letters 244, 367-372.

Robredo, B., Singh, K.V., Baquero, F., Murray, B.E., Torres, C., 1999. From *vanA Enterococcus hirae* to v*anA Enterococcus faecium*: a study of feed supplementation with avoparcin and tylosin in young chickens. Antimicrob Agents Chemother 43, 1137-1143.

Rubio, R., Jofré, A., Martín, B., Aymerich, T., Garriga, M., 2014. Characterization of lactic acid bacteria isolated from infant faeces as potential probiotic starter cultures for fermented sausages. Food Microbiol 38, 303-311.

Salyers, A.A., Gupta, A., Wang, Y., 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology 12, 412-416.

Sharma, P., Tomar, S.K., Goswami, P., Sangwan, V., Singh, R., 2014. Antibiotic resistance among commercially available probiotics. Food Research International 57, 176-195.

Snydman, D.R., 2008. The safety of probiotics. Clinical Infectious Diseases 46, S104-S111.

Sutcliffe, J., Grebe, T., Tait-Kamradt, A., Wondrack, L., 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 40, 2562-2566.

Vakulenko, S.B., Mobashery, S., 2003. Versatility of aminoglycosides and prospects for their future. Clinical Microbiology Reviews 16, 430-450.

Van de Klundert, J.A.M., Vliegenthart, J.S., 1993. PCR detection of genes coding for aminoglycoside-modifying enzymes. Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, DC, 547-552.

Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M., Entenza, J.M., Moreillon, P., Wind, R.D., Knol, J., Wiertz, E., Pot, B., Vaughan, E.E., Kahlmeter, G., Goossens, H., 2008. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends in Food Science & Technology 19, 102-114.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H., 2004. Development of a multiplex PCR for the detection of *asa*1, *gel*E, *cyl*A, *esp*, and *hyl* genes in *enterococci* and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. J Clin Microbiol 42, 4473-4479.

Wilson, J., Schurr, M., LeBlanc, C., Ramamurthy, R., Buchanan, K., Nickerson, C., 2002. Mechanisms of bacterial pathogenicity. Postgraduate Medical Journal 78, 216-224.

Zhang, X., Zhang, T., Fang, H.H.P., 2009. Antibiotic resistance genes in water environment. Applied Microbiology and Biotechnology 82, 397-414.

CHAPTER 4 - TECHNOLOGICAL PROPERTIES OF LACTIC ACID BACTERIA OBTAINED FROM A DAIRY PRODUCTION ENVIRONMENT AND DEVELOPMENT OF A FERMENTED MILK WITH THE BENEFICIAL STRAIN *LACTOBACILLUS CASEI* MRUV6

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In preparation for submission

Abstract

In a previous study, 15 lactic acid bacteria strains were isolated from a dairy environment, identified as *Lactobacillus* (n = 11; *Lb. casei* MSI1, *Lb. casei* MSI5, *Lb. casei* MRUV1, *Lb. casei* MRUV6, *Lb. acidophilus* MVA3, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSI3, *Lb. harbinensis* MSIV2, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5 and *Lb. plantarum* MSI2), *Pediococcus* (n = 2; *P. pentosaceus* MLEV8 and *P. acidilactici* MSI7) and *Weissella* (n = 2; *W. paramesenteroides* MRUV3 and *W. paramesenteroides* MSAV5) and selected due to their beneficial and safety potential. The present study aimed to characterize the technological properties of these strains and to select one to develop a fermented milk. All strains presented acidification capacity, reaching pH values between 0.73 and 2.11 in 24 hours: *Lb. casei* MRUV6 presented the highest acidification ability (pH 2.11 after 24 h). All strains were able to produce diacetyl at 37 °C, except by *Lb. casei* MSI1, *Lb. harbinensis* MSI3, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5 and *W. paramesenteroides* MRUV3. All strains were able to produce exopolysaccharides, and only two strains presented proteolytic activity (*Lb. casei* MSI5 and *W. paramesenteroides* MSAV5). Based on this characterization, *Lb. casei* MRUV6 was selected for producing fermented milk, stored at 4 and 10 °C and monitored until 35 days of shelf life. Samples were subjected to phenotypical and molecular methods to evaluate the presence of *Lb. casei* MRUV6 (conventional plating and RT-PCR, by checking the expression of *gapdh*, a housekeeping gene) and to verify the expression of *bsh* gene, related to resistance to bile salts (RT-PCR). *Lb. casei* MRUV6 population was stable during storage period at 4 and 10 °C at levels around 9.9 log CFU/g, and by monitoring the expression of GAPDH. However, *bsh* gene was not expressed during storage period. The study demonstrated the potential use of the beneficial strain *Lb. casei* MRUV6 isolated from a dairy environment for the production of a fermented milk product, and its stability during storage at 4 and 10 °C.

Keywords: benefic bacteria, technological potential, fermented milk, viability, real time PCR.

1. Introduction

The study of beneficial properties of lactic acid bacteria (LAB) has being considered a trend in the food industry in the last decades. Among different positive aspects that these bacteria can provide and deliver to consumers, the characterization of their probiotic properties is of particular interest from nutritional and medical aspects. Probiotic bacteria are defined as living organisms that benefit consumer health when ingested in appropriate concentrations (FAO/WHO, 2002). Many studies are being conducted to elucidate their mechanisms of activity and how to use them effectively in diseases prevention and treatment for humans and other animals (Ceapa et al., 2013; Scott et al., 2011). However, it is important to underline that these cultures need to be able to keep their viability (presenting minimum populations of 10^5-10^7 CFU/g) during the shelf-life of food products (Davidson et al., 2000; FAO/WHO, 2006).

Fermented milk products are the most commonly studied and applied food commodities as vectors for probiotic cultures delivery. Dairy products with added probiotic cultures are the highly explored alternatives that can best serve the consumer in their search for different and beneficial products for the promotion of health and well-being (Farnworth, 2008; Fontana et al., 2013). However, for the development of new products, several aspects must be addressed, including beneficial, technological and safety properties of the selected LAB, sensorial analysis of the final products, among others. The technological potential of new beneficial LAB should be analyzed in order to be incorporated into products for human consumption and to ensure their positive behavior in the final product (Fontana et al., 2013; Karimi et al., 2012). Several studies have demonstrated the potential of these products, based on the beneficial features of added LAB cultures with direct effects on consumer health (Karimi et al., 2012; Lollo et al., 2013).

Probiotic bacteria added to any specific food must be present at adequate concentrations during the entire shelf-life of the product, demanding proper monitoring by reliable methods to ensure the beneficial aspect of the food (Mani-Lopez et al., 2014; Tripathi and Giri, 2014; Yerlikaya, 2014). Conventional approaches for enumeration of microorganisms, including LAB, can be improved by molecular methods, leading to shorter time for final and trustable results (Ilha et al., 2016; Sarvari et al., 2014). Molecular methods are based on the direct analysis of DNA and/or RNA extracted from the food matrix (Achilleos and Berthier, 2013; Rodríguez et al., 2012) and applying different techniques can answer about presence and quantity of the monitored beneficial

cultures. PCR, Real Time-PCR, and quantitative RT-PCR are just few examples of methods that can be applied in these studies (Furet et al., 2004; Herbel et al., 2013).
The present study aimed to characterize the technological properties of 15 LAB strains isolated from a dairy production environment, previously characterized as beneficial, and to select a strain candidate to be considered as starter culture in the production of fermented milk and monitored during the shelf-life.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

In a previous study (Chapter 2 of this thesis), a culture collection composed of 15 isolates selected on preliminary screening from 500 isolates, obtained from a dairy environment, was characterized as beneficial by phenotypical and molecular methods. The isolates were identified as *Lactobacillus* spp. (n = 11), *Pediococcus* spp. (n = 2) and *Weissella* spp. (n = 2) by sequencing of 16s rRNA and grouped according to rep-PCR and RAPD PCR (Chapter 2 of this thesis). Based on these characteristics, *Lb. casei* MSI1, *Lb. casei* MSI5, *Lb. casei* MRUV1, *Lb. casei* MRUV6, *Lb. acidophilus* MVA3, *Lb. nagelli* MSIV4, *Lb. harbinensis* MSI3, *Lb. harbinensis* MSIV2, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5, *Lb. plantarum* MSI2, *P. pentosaceus* MLEV8, *P. acidilactici* MSI7, *W. paramesenteroides* MRUV3 and *W. paramesenteroides* MSAV5 were selected for the present study. The isolates were stored in de Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) supplemented with 25% (v/v) glycerol. For use, stock cultures were streaked on MRS agar (Oxoid), incubated at 37 °C for 24 h, and then isolated colonies were transferred to MRS broth (Oxoid) and incubated at 37 °C for 24 h.

2.2. Technological potential

2.2.1. Acidifying ability

The strains were grown in MRS broth (Oxoid) and incubated at 37 °C for 24 h. Culture aliquots (1 % v/v) were inoculated in 10 mL of reconstituted skim milk powder (Difco, 10 % w/v) and incubated at 37 °C for 24 h. The pH was measured after 6 and 24 h of incubation using pH meter (HI 221, Hanna Instruments, São Paulo, SP, Brazil). The averages of two repetitions were determinate and acidification rate was calculated as

ΔpH (ΔpH= pH zero time – pH6 or 24h time). The ΔpH values after 6 h (ΔpH6) and 24 h (ΔpH24) were used to compare the acidification activity of the strain (Morandi et al., 2011).

2.2.2. Diacetyl production

Aliquots (1% v/v) of the strains were inoculated in 10 mL of reconstituted skim milk powder (Difco, 10 % w/v) and incubated at 37 °C for 24 h. Then, 1 mL of each cell culture was added 0.5 mL α-naftol (1% w/v) and KOH (16% w/v) and incubated at 37 °C for 10 min. The diacetyl production was determined by red ring formed at the top of the tubes (Dal Bello et al., 2012).

2.2.3. Exopolysaccharides formation

Aliquots (1 % v/v) of the strains were inoculated in 10 mL of reconstituted skim milk powder (Difco, 10 % w/v) and incubated at 37 °C for 24 h. Exopolysaccharides formation from lactose was determined qualitatively by measuring the degree of wire forming (Cogan and Accolas, 1996; Dal Bello et al., 2012).

2.2.4. Extracellular proteolytic activity

The assessment of proteolytic activity was determined using the protocol described by Franciosi et al. (2009) and Dal Bello et al. (2012). Aliquots of 1 µL of the strains were punctually inoculated in agar (2% w/v) supplemented with reconstituted skim-milk powder (Difco, 10% w/v) and incubated at 37 °C for 4 days. Positive result was indicated by translucent halo around the colonies: halos with up to 2 mm radius are ranked as +, between 2 and 4 mm as ++ and above 4 mm as +++ (Dal Bello et al., 2012).

2.3 Fermented milk

Strain selection and fermented milk production

Lb. casei MRUV6 was recovered in MRS at 37 °C for 18 h. Then, the obtained culture was diluted until turbidity similar to standard 3 from McFarland scale, corresponding to approximately 9.0 x 10^8 colony forming units per mL (CFU/mL).

The fermented milk production was done according to Tamine (1991). The preparation was made from 1 L of reconstituted skim milk powder (Difco, 10 % w/v), heated at 90 °C for 5 min, and cooled to 37 °C. Then, 20 mL of the previously prepared *Lb. casei*

MRUV6 culture was added and incubated at 37 °C for 7 days, being the acidity monitored daily by NaOH 0.8 N titration until 1.7%. The obtained fermented milk was distributed into sterile plastic flasks (100 mL) and stored at 4 °C and 10 °C for 35 days.

Lb. casei MRUV6 populations monitoring during storage

Samples of the prepared fermented milks were collected during storage at the following times: 0 h (just after the preparation of fermented milk), and after 7, 14, 21, 28 and 35 days. Each sample was ten-fold diluted using saline (NaCl0.85 %, w/v). Selected dilutions were pour plated on MRS-V agar (MRS agar supplemented with 10 mg/L (final concentration) of filter sterilized vancomycin, 0.22 µm) in duplicates, according to Colombo et al. (2014). Plates were incubated at 37 °C under aerobic conditions for 48 h. After incubation, the colonies were enumerated and the results were expressed as CFU/g.

Fermented milk production and microbiological analyses were conducted in three independent repetitions. To verify the viability of the *Lb. casei* MRUV6 on fermented milk, the count of analysed strain was converted to $\log_{10}$ and compared by analysis of variance (ANOVA) ($p < 0.05$).

Lb. casei MRUV6 resistance to bile during storage

Dilutions from collected samples of fermented milk obtained for *Lb. casei* MRUV6 enumeration were also pour plated in duplicates in MRS-B agar (MRS agar (BD) supplemented with bile salts (1.5%, w/v, Sigma), and incubated at 37 °C under aerobic conditions for 48 h. After incubation, the colonies were enumerated and the results were expressed as CFU/g, and then converted to log CFU/g and compared by ANOVA ($p < 0.05$) to check significant differences among incubation periods and with the *Lb. casei* MRUV6 counts obtained previously in MRS-V.

Expression of bile salt hydrolase (bsh) gene

RNA isolation and cDNA synthesis. Aliquots of fermented milk samples obtained for *Lb. casei* MRUV6 monitoring were subjected to RNA extraction, without any previous treatment (T1), and after being diluted in MRS broth (BD) supplemented with bile salts (1.5%, w/v, Sigma) followed by incubation at 37 °C for 4 h (T2). Then, treated samples were centrifugated at 4,000 *x g* for 5 min at 4 °C, and the total RNA were extracted by using Total RNA Purification Kit (NorgenBiotek Corporation, Thorold, ON, Canada) and RNase free DNase-I (Thermo Fisher Scientific, Waltham, MA, USA) to remove the

genomic DNA. cDNA was synthesized with 0.2 µl of total RNA (1 µg/µl), 1 µl DNase (rDNAse I), 5 µl of buffer (10x DNase I Buffer) 0.5 µl of dNTP´s and 44 µl of RNase Free ddH2O. After 30 min at 37 °C, DNase inactivation was added and incubated for 2 min, harvested by centrifugation at 10,000 *x g* for 1.5 min and the supernatant was kept at 4 °C. Reverse transcriptions (RT) were performed with 1 µl of total RNA and 1 µl MMLVRT Enzyme (Thermo Fisher Scientific, Waltham, MA, EUA). The RT reaction condition was as follows: 25 °C for 10 min, 37 °C for 50 min, 70 °C for 20 min.

Primers. The primers chosen for this work were based on the reported genome sequences of *Lb. casei* (ATCC 334, BL23 and DSM20011) (Table 1). For RT-PCR analysis, GAPDH, RT-GAPDH and RT-pla2 were selected to verify the viability of *Lb. casei* MRUV6 and to be considered as endogenous control, and BSHQ was selected to check the expression of bile salt hydrolase, related to bile salts tolerance.

Table 1. PCR primers

Primer	Target gene	Sequence (5´ to 3´)	References
BSHQ	*bsh*	F: ATAGTCCTGAATACGGGTGG R: GATGGTGATGTGTAATCGCC	Zhang et al. (2009)
GAPDH	*gapdh*	F: GGCTATCGGTTTGGTTATCC R: TGTTTTCGGTGTGCTTCTTG	Zhang et al. (2009)
RT-GAPDH	*gapdh*	F: GAAGCTTTGATGACTACCGTT R: CTTTACCGACAGCCTTAGCAG	Wang et al. (2015)
RT-pla2	*pla2*	F: ACAATGTTGATCCCGTTGTCC R: CGCAATAATTCCCATACACCAG	Wang et al.(2015)

Real time PCR. The RT-PCR was performed according to Wang (2015) with minor modifications. The analysis had been carried out on Rotor Gene Q (Qiagen, Hilden, Germany). The total volume of the PCR was 20 µL, and each reaction mixture contained SYBR Premix ExTaq II PCR buffer and 0.4 mM of each forward and reverse primer. Amplification was conducted as following: 94 °C for 30 s, followed by 40 cycles at 63 °C for 30 s and at 72 °C for 30 s. Each run was completed with a melting curve analysis to confirm the specificity of the amplifications. A negative amplification control of a sample containing no DNA was used. GAPDH was selected as an internal control for normalizing the amount of RNA added to the reaction of reverse transcription. Individual real-time PCR reactions were carried out in three repetitions and duplicates for each gene.

3. Results and discussion

The results for technological potential of 15 LAB strains are shown in Table 2. All strains showed acidification capacity, reaching pH values between 0.73 and 2.11 in 24 h periods (Table 2). *Lb. casei* MRUV6 was the strain that presented the highest acidifying ability, reaching pH 2.11 after 24 h. Although acid production by LAB is dependent strain, as already reported in other studies, in general the high acidification capacity is directly related to the rapid acidification of the raw material by the production of organic acids, mainly lactic acid (Dal Bello Et al., 2012; Nieto-Arribas et al., 2009; Pingitore et al., 2012; Piraino et al., 2008).

Diacetyl production is a desired feature of a starter culture, once it improves the aromatic and organoleptic characteristics of the fermented dairy products (Passerini et al., 2013). The obtained results for diacetyl production showed that 10 strains were able to produce this compound, being only *Lb. casei* MSI1, *Lb. harbinensis* MSI3, *Lb. fermentum* SIVGL1, *Lb. plantarum* MLE5 and *W. paramesenteroides* MRUV3 unable to produce diacetyl at 37 °C. The result observed for the production of diacetyl was satisfactory, since *Lb. casei* MRUV6 was able to produce this aromatic compound, being considered a moderate producer. For the food industry, the diacetyl compound is appreciated in the fermentation of dairy products, improving the organoleptic characteristics of dairy products (Samet-Bali and Attia, 2012; Smit et al., 2005).

All strains presented positive results for exopolisaccharides production showing that they are able to improve the texture of dairy food products, increasing its viscosity and firmness (Dal Bello et al., 2012). Exopolisaccharides synthesized by BAL strains plays important role in the manufacture of fermented dairy products (Table 2). Only two strains presented proteolytic activity (*Lb. casei* MSI5 and *W. paramesenteroides* MSAV5), while all others were negative (Table 2). LAB cultures does not necessarily have all desirable technological properties, because even with just a few of these characteristics, it is already enough for the production of dairy products, for example, being used as coadjutants for the production of flavorings and in the production of other fermented foods (Hassan and Frank, 2001; Crow et al., 2001).

Table 2. Technological potential results of 15 LAB strains.

LAB	Acidifying capacity		Dyacetyl	Exopolisaccharides	Proteolysis
	ΔpH6	ΔpH24			
Lactobacillus casei MSI1	0.47	0.96	-	+	-
Lb. casei MSI5	0.47	1.61	+	+	++
Lb. casei MRUV1	0.48	1.81	+	+	-
Lb. casei MRUV6	0.52	2.11	+	+	-
Lb. acidophilus MVA3	0.44	1.45	+	+	-
Lb. nagelli MSIV4	0.43	1.27	+	+	-
Lb. harbinensis MSI3	0.50	1.49	-	+	-
Lb. harbinensis MSIV2	0.40	1.72	+	+	-
Lb. fermentum SIVGL1	0.65	1.68	-	+	-
Lb. plantarum MLE5	0.22	0.73	-	+	-
Lb. plantarum MSI2	0.63	1.52	+	+	-
Pediococcus pentosaceus MLEV8	0.41	1.49	+	+	-
P. acidilactici MSI7	0.39	2.00	+	+	-
Weissella paramesenteroides MRUV3	0.52	1.86	-	+	-
W.paramesenteroides MSAV5	0.53	1.29	+	+	+

Halos with up to 2 mm radius are ranked as +, between 2 and 4 mm as ++ and above 4 mm as +++.

Lb. casei MRUV6 was selected as more promising candidate for this study based on its beneficial and safety characteristics (Chapters 2 and 3). Moreover, this strain presented promising technological properties, based on research in this study that can favor and allow its potential application in the production of fermented dairy products (Table 2). The technological potential results showed that the metabolic activity of *Lb. casei* MRUV6 is capable of influencing the development of desirable organoleptic properties, thus allowing the preservation and raising the nutritional value of these products (Alexandre et al., 2002; Galia et al., 2009). Based on obtained results, fermented milk was produced by using *Lb. casei* MRUV6 as mono starter culture and its populations were monitored during the shelf-life of this product (Table 3).

Lb. casei MRUV6 populations ranged from 9.72 to 9.96 log CFU/g in fermented milk as determined by plate count at 0, 7, 14, 21, 28 and 35 days after fermented milk preparation in 4 °C and 10 °C. The results of enumeration of *Lb. casei* MRUV6 for 35 days at both temperatures, showed no difference between 4 °C and 10 °C of storage. The temperature of 4 ° C was considered ideal for preservation of the fermented milk, however at 10 ° C it was also possible to observe that the product can be conserved in an ideal way, mimicking the conditions of trade (Brasil, 2011; Freitas et al., 2015). Over the 35 days analyzed, the strain remained stable at 9 log. Even though in some situations there were differences between both temperatures that were even significant, the counts showed that the populations were always above 7 log, which is desirable for beneficial BALs in fermented foods. *Lb. casei* MRUV6 remained stable throughout the experimental period, presenting a slight decrease between days 7 and 14, returning to stability around day 21. This is expected of foods added by beneficial bacteria, that they remain stable in the lag phase and do not multiply and can lead to deterioration of the product (Valero et al., 2012). This stability can be observed during the 35 days of experiment at both temperatures and that is to be expected from this type of experiment with LAB (Mani-López et al., 2014; Miranda et al., 2011; García-Cayuela et al., 2009; Furet et al., 2004).

Table 3. Mean counts of *Lb. casei*MRUV6 in fermented milk stored at 4 °C and 10 °C during 35 days in MRS-V agar and MRS-B (results are expressed as $\log_{10}$ CFU/g).

Time (days)	MRS-V		MRS-B		ANOVA
	4 °C	10 °C	4 °C	10 °C	
0	9.91 ± 0.05^{aA}	9.86 ± 0.01^{abA}	9.79 ± 0.00^{aB}	9.79 ± 0.00^{aB}	F=18.79; gl=3; p=0.001
7	9.82 ± 0.02^{bA}	9.75 ± 0.02^{cB}	9.72 ± 0.00^{cB}	9.72 ± 0.00^{cB}	F=37.64; gl=3; p<0.001
14	9.81 ± 0.00^{bA}	9.80 ± 0.03^{bcA}	9.73 ± 0.00^{bB}	9.73 ± 0.00^{dB}	F=22.96; gl=3; p<0.001
21	9.83 ± 0.01^{bA}	9.84 ± 0.07^{abA}	9.73 ± 0.00^{bB}	9.73 ± 0.00^{cdB}	F=9.42; gl=3; p<0.005
28	9.87 ± 0.05^{abA}	9.91 ± 0.00^{aA}	9.73 ± 0.00^{bB}	9.73 ± 0.00^{bcB}	F=43.50; gl=3; p<0.001
35	9.84 ± 0.02^{abB}	9.90 ± 0.01^{aA}	9.73 ± 0.00^{bC}	9.73 ± 0.00^{bC}	F=153.79; gl=3; p<0.001
ANOVA	F=4.91; gl=5; p=0.011	F=11.77; gl=5; p=0.00	F=753.49; gl=5; p<0.001	F=513.45; gl=5; p<0.01	

* Values followed by distinct upper-case letters are significantly different ($p < 0.05$). Lower-case letters are for comparison between times (columns) and upper case are for comparison between temperatures and means (lines).

Despite being observed some significant differences among *Lb. casei* MRUV6 counts from fermented milk stored at 4 and 10 °C (Table 3), after 7 and 35 days, it is possible to observe that the strain was viable at high concentrations. In addition, when comparing the counts at 4 °C and 10 °C, we identified that in the first the culture undergoes a slight reduction in their counts and, on the contrary, in the second temperature the culture can multiply when we compare day 0 with the others days. At both temperatures, the culture remains stable for up to 21 days, and there after begins to undergo reduction or growth over days depending on the storage temperature used. In fact, at 4 and 10 °C storage, *Lb. casei* was not supposed to growth. Studies with beneficial BAL demonstrate the stability of the cultures during the shelf-life of the product and this concentration of live beneficial bacteria is considered enough to exert health benefits (Ilha et al., 2015; Roy, 2005). This result corroborates with that already found by fermented milk with beneficial microorganisms and it is important for product storage and LAB strain viability (Lima et al., 2009; Sarvari et al., 2014).

gadph gene was selected as an internal control for normalizing the amount of RNA added to the reaction of reverse transcription. This gene has been chosen by other authors as endogenous control for RT PCR (Wang et al., 2015; Zhang et al., 2009). The real-time PCR results related to the *gapdh* were positive for the chosen strain, showing that at all times this gene was properly expressed. Based on these results, *gapdh* was chosen as reference, being able to indicate the viability of the culture added in the fermented milk. This expression was related to the RNA extracted directly from the fermented milk samples, not through any previous treatment.

The fermented milk samples were also plated in MRS-B, and the results of the counts are shown in Table 3. Although there are differences with the total counts, the results indicate the ability of *Lb. casei* MRUV6 to survive in a bile environment. There was a small reduction when we compared the counts obtained on MRS-V agar and MRS-B and a slight variation of populations throughout the storage. However, at both storage temperatures, *Lb. casei* MRUV6 was able to remain viable and at optimum concentrations to stabilize its consumer benefits. Considering the counts on MRS-B, there was a reduction of day 0 with 7 days and, in the others, it remained stable with similar counts. In all treatments and counts, the differences were always significant ($p < 0.05$), indicating some difficult by the strain in growing in this culture media added with bile, but still its ability in surviving in such condition.

The data presented in Table 3 confirm what was expected by the culture contact with bile, which is in accordance with Chapter 2 of this thesis, where the results of

deconjugation of bile salts show the ability of *Lb. casei* MRUV6 to resist bile salts. The deconjugation of bile by *Lb. casei* MRUV6 after 0, 7, 14, 21, 28 and 35 days incubation at 4 °C and 10 °C was measured in terms of its viability in MRS-B after milk fermentation. This could indicate that *Lb. casei* MRUV6 is capable of deconjugate bile salts during growth by producing certain bile salt hydrolases. The strain showed viability after milk fermentation during 35 days of storage (data not shown). In chapter 2 of this thesis, all 15 selected LAB strains were able to survive in bile concentration and deconjugating bile salts, what is expected by LAB and other intestinal bacteria that have been reported to be involved in transformation reactions and catalyzing hydrolysis of conjugated bile salts (Zhang et al. 2009; Begley et al., 2006). However, it is important to underline that survive in presence in bile salts and deconjugation are two different phenomena. Biochemical assay done in Chapter 2 of this thesis indicated that *Lb. casei* MRUV6 was able to survive in presence of bile salt and also deconjugated bile salts during the experiment, which also exhibited considerable activity; similarly, other authors found similar results for beneficial bacteria (Zhang et al., 2009; Brashears et al., 1998). The most prevalent mechanisms that moderate resistance in several bacterial genera are the active efflux of bile acids/salts (Pfeiler and Klaenhammer, 2009; Bustos et al., 2011; Ruiz et al., 2012a,b), bile salt hydrolysis (Kumar et al., 2006; Lambert et al., 2008), and changes in the architecture/composition of cell membrane and cell wall (Gómez-Zavaglia et al., 2002; Taranto et al., 2003; Ruiz et al., 2007). The bacterial mechanisms of resistance to bile can be considered multifactorial, implying in a great variety of processes aimed at detoxification by bile and against the deleterious effect of these salts on bacterial structures(Ruiz et al., 2013).

bsh has been the most used gene in these studies of resistance to bile and deconjugation of bile salts by *Lactobacillus* sp (Zhang et al., 2009; Wang et al., 2015). Some regions of *bsh* have been sequenced and characterized from *Lactobacillus* strains, but there is just little information about this gene in *Lb. casei* strains (Zhang et al., 2009; Tanaka et al., 1999). Studies have shown that although most species of *Lactobacillus* present *bsh*, some species do not express its products. In our study, *Lb. casei* MRUV6 presented *bsh* by PCR, but RT-PCR results indicated absence of *bsh* expression in the RNA extracted directly from the sample. Thus, fermented milk samples were treated with bile in order to try to stimulate *bsh* expression; even with after this treatment, expression was not detected. *gaphd* gene was properly expressed with GAPDH primer even after such treatment, indicating that the strain kept its viability. Even though *bsh* is not expressed under the conditions provided by the treatment, the culture is able to grow in medium

with bile, as shown in the results of Table 3. Despite *bsh*, other factors can be activated to enable the viability of LAB strains to survive in an environment with bile. As *Lb. casei* MRUV6 growth in an environment with bile (Table 3, chapter 2), this strain might present alternatives for this ability. Some studies describe different pathways for resistance to bile and deconjugation of bile salts, in addition to *bsh* expression, and a number of bile resistance mechanisms (Grill et al., 1995; Jones et al., 2008; Kim et al., 2005; McAuliffe et al., 2005). Despite being considered relevant for lactobacilli and bifidobacterial, little information is available about *bsh* activity (Bi et al., 2016; Xiong et al., 2017).

The absence of *bsh* expression by *Lb. casei* MRUV6 can be related to different degrees of BSH activity by bacteria (Zhang et al., 2009). The advantage for bacteria to tolerate bile salts by BSH activity can be controversial, what is dependent of the bile concentration in the environment (Begley et al., 2005, 2006). Despite being a positive feature for beneficial bacterial, the resistance to bile can be considered a signal for virulence and adaptive behavior of bacteria (Gunn, 2000). An understanding of the resistance and response of bacteria to bile may assist the development of novel therapeutic, prevention, and diagnostic strategies to treat enteric and extra intestinal infections.

4. Conclusion

The obtained results indicated the technological potential of LAB isolated from a dairy production environment, allowing the selection of the strain *Lb. casei* MRUV6 for the production of a fermented milk, due to its additional beneficial and safety properties. *Lb. casei* MRUV6 populations were stable and active during all storage period of fermented milk. Monitoring *gapdh* expression indicated the viability of *Lb. casei* MRUV6 during storage, but *bsh* was not expressed, indicating alternative mechanisms for bile resistance by the beneficial strain.

References

Achilleos, C., Berthier, F., 2013. Quantitative PCR for the specific quantification of *Lactococcus lactis* and *Lactobacillus paracasei* and its interest for *Lactococcus lactis* in cheese samples. Food Microbiology 36, 286-295.

Alexandre, D.P., Silva, M.R., Souza, M.R., Santos, W.L.M., 2002. Atividade antimicrobiana de bactérias lácticas isoladas de queijo-de-minas artesanal do Serro (MG) frente a microrganismos indicadores. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 54, 424-428.

Bas, A., Forsberg, G., Hammarstrom, S., Hammarstrom, M.L., 2004. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. Scandinavian Journal of Immunology 59, 566-573.

Begley, M., Hill, C., Gahan, C.G.M., 2006. Bile salt hydrolase activity in probiotics. Applied and Environmental Microbiology 72, 1729-1738.

Begley, M., Gahan, C.G., Hill, C., 2005. The interaction between bacteria and bile. FEMS Microbiology Reviews 29, 625-651.

Bi, J., Liu, S., Du, G., Chen, J., 2016. Bile salt tolerance of *Lactococcus lactis* is enhanced by expression of bile salt hydrolase thereby producing less bile acid in the cells. Biotechnology Letters, 38, 659-665.

Bustos,A.Y., Raya,R., De Valdez,G.F., Taranto,M.P., 2011. Efflux of bile acids in *Lactobacillus reuteri* is mediated by ATP. Biotechnology Letters33, 2265–2269.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa nº 62. Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, o Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, o Regulamento Técnico de Identidade e Qualidade de Leite Pasteurizado e o Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel. Brasília: MAPA, 2011.

Ceapa, C., Wopereis, H., Rezaiki, L., Kleerebezem, M., Knol, J., Oozeer, R., 2013. Influence of fermented milk products, prebiotics and probiotics on microbiota composition and health. Best Practice & Research Clinical Gastroenterology 27, 139-155.

Cerning, J., Bouillanne, C., Landon, M., Desmazeaud, M., 1992.Isolation and characterization of exopolysaccharides from slime-forming mesophilic lactic acid bacteria. Journal of Dairy Science 75, 692-699.

Cogan, T.M., Accolas, J., 1996. Dairy starter cultures.VCH Publishers Inc.

Colombo, M., Castilho, N.P.A., Todorov, S.D., Nero, L.A., 2017. Beneficial properties of lactic acid bacteria naturally present in dairy production systems. Journal of Food Science and Technology, *in submission.*

Colombo, M., Oliveira, A.E.Z., Carvalho, A.F., Nero, L.A., 2014. Development of an alternative culture medium for the selective enumeration of *Lactobacillus casei* in fermented milk. Food Microbiology 39, 89-95.

Crow, V., Curry, B., Hayes, M., 2001. The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. International Dairy Journal 11, 275-283.

Dal Bello, B., Cocolin, L., Zeppa, G., Field, D., Cotter, P.D., Hill, C., 2012. Technological characterization of bacteriocin producing *Lactococcus lactis* strains employed to control *Listeria monocytogenes* in Cottage cheese. International Journal of Food Microbiology 153, 58-65.

Davidson, R.H., Duncan, S.E., Hackney, C.R., Eigel, W.N., Boling, J.W., 2000. Probiotic culture survival and implications in fermented frozen yogurt characteristics. Journal of Dairy Science 83, 666-673.

FAO/WHO, 2002. Working group on drafting guidelines for the evaluation of probiotics in food, Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada.

FAO/WHO, 2006. Health and nutritional properties and guidelines for evaluation. FAO/WHO Paper 85.

Farnworth, E.R.T., 2008. Handbook of fermented functional foods.CRC press, Boca Raton, Florida, USA.

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J.,Munoz-Quezada, S., Gil, A., 2013. Sources, isolation, characterization and evaluation of probiotics. The British Journal of Nutrition 109 Suppl 2, S35-S50.

Franciosi, E., Settanni, L., Cavazza, A., Poznanski, E., 2009. Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. International Dairy Journal 19, 3-11.

Freitas, R., Miranda, R.O., Netto, G.G., Nero, L.A., Carvalho, A.F., 2015. Interference of different storage temperatures in the dynamics of probiotic *Bifidobacterium* spp. and *Streptococcus thermophilus* starter cultures in fermented milk. Arquivo do Instituto Biológico, 82, 1-4.

Furet, J., Quénée, P., Tailliez, P., 2004. Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. International Journal of Food Microbiology 97, 197-207.

Galia, W., Perrin, C., Genay, M., Dary, A., 2009. Variability and molecular typing of *Streptococcus thermophilus* strains displaying different proteolytic and acidifying properties. International Dairy Journal 19, 89-95.

García-Cayuela, T., Tabasco, R., Peláez, T.R., 2009. Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidiummonoazideandreal-time PCR. International Dairy Journal, 19, 405-409.

Gómez-Zavaglia, A., Kociubinski, G., Pérez, P., De Antoni, G., 2002. Effect of bile on the lipid composition and surface properties of bifidobacteria. Journal of Applied Microbiology 93, 794–799.

Grill, J., Schneider, F., Crociani, J., Ballongue, J., 1995. Purification and characterization of conjugated Bile Salt Hydrolase from *Bifidobacterium longum* BB536. Applied and Environmental Microbiology 61, 2577-2582.

Gunn, J.S., 2000. Mechanisms of bacterial resistance and response to bile. Microbes and Infection 2, 907-913.

Hassan, A.N., Frank, J.F., 2001. Starter cultures and their use. Food Science and Technology, 151-206.

Herbel, S.R., Lauzat, B., von Nickisch-Rosenegk, M., Kuhn, M., Murugaiyan, J., Wieler, L.H., Guenther, S., 2013.Species-specific quantification of probiotic lactobacilli in yoghurt by quantitative real-time PCR. Journal of Applied Microbiology 115, 1402-1410.

Huggett, J., Dheda, K., Bustin, S., Zumla, A., 2005. Real-time RT-PCR normalization: strategies and considerations. Genes and Immunity 6, 279-284.

Ilha, E.C., Scariot, M.C., Treml, D., Pereira, T.P., Sant′Anna, E.S., Prudêncio, E.S., Arisi, A.C.M., 2015. Comparison of real-time PCR assay and plate count for *Lactobacillus paracasei* enumeration in yoghurt. Annals of Microbiology 66, 597-606.

Jolly, L., Vincent, S.J.F., Duboc, P., Neeser, J., 2002. Exploiting exopolysaccharides from lactic acid bacteria.Antonie van Leeuwenhoek 82, 367-374.

Jones, B.V., Begley, M., Hill, C., Gahan, C.G.M., Marchesi, J.R., 2008. Functional and comparative metagenomic analysis of Bile Salt Hydrolase activity in the human gut microbiome. Proceedings of the National Academy of Sciences of the United States of America 105, 13580-13585.

Karimi, R., Mortazavian, A.M., Amiri-Rigi, A., 2012. Selective enumeration of probiotic microorganisms in cheese. Food Microbiology 29, 1-9.

Kim, G.B., Brochet, M., Lee, B.H., 2005. Cloning and characterization of a Bile Salt Hydrolase (BSH) from *Bifidobacterium adolescentis*. Biotechnology Letters 27, 817-822.

Kumar, R.S., Brannigan, J.A., Prabhune, A.A., Pundle, A.V., Dodson, G.G., Dodson, E.J., 2006. Structural and functional analysis of a conjugated bile salt hydrolase from *Bifidobacterium longum* reveals an evolutionary relationship with penicillin Vacylase. The Journal of Biological Chemistry 281, 32516–32525.

Lambert, J.M., Bongers, R.S., de Vos, W.M., Kleerebezem, M., 2008. Functional analysis of four bile salt hydrolase and penicillinacylase family members in *Lactobacillus plantarum* WCFS1. Applied and Environmental Microbiology 74, 4719–4726.

Langa, S., Martín-Cabrejas, I., Montiel, R., Landete, J.M., Medina, M., Arqués, J.L., 2014. Combined antimicrobial activity of reuterin and diacetyl against foodborne pathogens. Journal of Dairy Science 97, 6116-6121.

Lima, K.G.C., Kruger, M.F., Behrens, J., Destro, M.T., Landgraf, M., Franco, B.D.G.M., 2009. Evaluation of culture media for enumeration of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium animalis* in the presence *of Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. LWT - Food Science and Technology 42, 491-495.

Lollo, P.C.B., Moura, C.S., Morato, P.N., Cruz, A.G., Castro, W.F., Betim, C.B., Nisishima, L., Assis, F.F.J., Maróstica, M., Fernandes, C.O., 2013. Probiotic yogurt offers higher immune-protection than probiotic whey beverage. Food Research International 54, 118-124.

Mani-Lopez, E., Palou, E., Lopez-Malo, A., 2014. Probiotic viability and storage stability of yogurts and fermented milks prepared with several mixtures of lactic acid bacteria. Journal of Dairy Science 5, 2578-2590.

McAuliffe, O., Cano, R.J., Klaenhammer, T.R., 2005. Genetic analysis of two Bile Salt Hydrolase activities in *Lactobacillus acidophilus* NCFM. Applied and Environmental Microbiology 71, 4925-4929.

Miranda, R.O., Neto, G.G., de Freitas, R. de Carvalho, A.F., Nero, L.A., 2011. Enumeration of bifidobacteria using Petrifilm™ AC in pure cultures and in a

fermented milk manufactured with a commercial culture of *Streptococcus thermophilus*. Food Microbiology 28, 1509-1513.

Morandi, S., Brasca, M., Lodi, R., 2011. Technological, phenotypic and genotypic characterization of wild lactic acid bacteria involved in the production of Bitto PDO Italian cheese. Dairy Science & Technology 91, 341-359.

Nieto-Arribas, P., Seseña, S., Poveda, J.M., Palop, L.l., & Cabezas, L., 2009. Genotypic and technological characterization of *Lactococcus lactis* isolates involved in processing of artisanal Manchego cheese. Journal of Applied Microbiology 107, 1505-1517.

Passerini, D., Laroute, V., Coddeville, M., Le Bourgeois, P., Loubière, P., Ritzenthaler, P., Cocaign-Bousquet, M., Daveran-Mingot, M., 2013. New insights into *Lactococcus lactis* diacetyl- and acetoin- producing strains isolated from diverse origins. International Journal of Food Microbiology 160, 329-336.

Pingitore, E.V., Todorov, S.D., Sesma, F., & Franco, B.D.G.M., 2012. Application of bacteriocinogenic *Enterococcus mundti* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese. Food Microbiology, 32, 38-47.

Piraino, P., Zotta, T., Ricciardi, A., McSweeney, P.L.H., & Parente, E., 2008. Acid production, proteolysis, autolytic and inhibitory properties of lactic acid bacteria isolated from pasta filata cheeses: A multivariate screening study. International Dairy Journal 18, 81-92.

Pfeiler,E.A., Klaenhammer,T.R., 2009. Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. Applied and Environmental Microbiology 75, 6013–6016.

Rodríguez, A., Córdoba, J.J., Gordillo, R., Córdoba, M.G., Rodríguez, M., 2012. Development of two Quantitative Real-Time PCR methods based on SYBR Green and TaqMan to quantify sterigmatocystin-producing molds in foods. Food Analytical Methods 5, 1514-1525.

Roy, D., 2005.Technological aspects related to the use of bifidobacteria in dairy products. Lait 85, 39–56.

Ruiz, L., Margolles, A., Sánchez, A., 2015. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. Frontiers in Microbiology 4, 1–8.

Ruiz, L., Zomer, A., O'Connell-Motherway, M., van Sinderen, D., Margolles, A., 2012b. Discovering novel bile protection systems in *Bifidobacterium breve*

UCC2003 through functional genomics. Applied Environmental Microbiology 78, 1123–1131.

Ruiz, L., Sánchez, B., Ruas-Madiedo, P., de los Reyes-Gavilán, C.G., Margolles, A., 2007. Cell envelope changes in *Bifidobacterium animalis* ssp. *lactis* as a response to bile. FEMS Microbiological Letters 274, 316–322.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W.M., Fondén, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S., Mattila-Sandholm, T., 1998. Demonstration of safety of probiotics - a review. International Journal of Food Microbiology 44, 93-106.

Samet-Bali, O., Attia, H., 2012. Characterization of typical Tunisian fermented milk, rayeb. African Journal of Biotechnology, 11, 6744-6749.

Sarvari, F., Mortazavian, A.M., Fazei, M.R., 2014. Biochemical characteristics and viability of probiotic and yogurt bacteria in yogurt during the fermentation and refrigerated storage. Applied Food Biotechnology 1, 55-61.

Scott, K.P., Duncan, S.H., Louis, P., Flint, H.J., 2011. Nutritional influences on the gut microbiota and the consequences for gastrointestinal health. Biochemical Society Transactions 39, 1073.

Settanni, L., Moschetti, G., 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiology 27, 691-697.

Shiby, V.K., Mishra, H.N., 2013. Fermented milks and milk products as functional foods - a review. Critical Reviews in Food Science and Nutrition 53, 482-496.

Smit, G., Smit, B.A., Engels, W.J.M., 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS – Microbiology Reviews, 29, 591-610.

Tamine, A.Y.R., R. K., 1991. Yogurte: ciência e tecnologia. Acribia. Ed. Zaragoza, 367.

Taranto, M.P., Murga, M.L.F., Lorca, G., De Valdez, G.F., 2003. Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. Journal of Applied Microbiology 95, 86–91.

Tripathi, M.K., Giri, S.K., 2014. Probiotic functional foods: Survival of probiotics during processing and storage. Journal of Functional Foods 9, 225-241.

Valero, A., Carrasco, E., Garcia-Gimeno, R., 2012. Principles and methodologies for the determination of shelf life in foods. In: Eissa AA (ed) Trends in vital food and control engineering. Intech, Rijeka.

Wang, A.N., Yi, X.W., Yu, H.F., Dong, B., Qiao, S.Y., 2009. Free radical scavenging activity of *Lactobacillus fermentum in vitro* and its antioxidative effect on growing–finishing pigs. Journal of Applied Microbiology 107, 1140-1148.

Wang, H., Zhang, L., Shi, G., 2015. Secretory expression of a phospholipase A2 from *Lactobacillus casei* DSM20011 in *Kluyveromyces lactis*. Journal of Bioscience and Bioengineering 120, 601-607.

Xiong, Z.Q., Wang, Q.H., Kong, L.H., Song, X., Wang, G.Q., Xia, Y.J., Zhang, H., Sun, Y, Ai, L.Z., 2017. Short communication: Improving the activity of bile salt hydrolases in *Lactobacillus casei* based on in silico molecular docking and heterologous expression. Journal of Dairy Science, 100, 975-980.

Yerlikaya, O., 2014. Starter cultures used in probiotic dairy product preparation and popular probiotic dairy drinks. Food Science and Technology 34, 221-229.

Zhang, W.Y., Wu, R.N., Sun, Z.H., Sun, T.S., Meng, H., Zhang, H.P., 2009. Molecular cloning and characterization of bile salt hydrolase in *Lactobacillus casei* Zhang. Annals of Microbiology 59, 721-726.

APPENDIX

Table 1. Screening for searching of LAB isolates in dairy environment.

Sample	Culture media	Initial isolates	Positive Gram	Negative catalase	pH 2.0	Bile 3%
Milk	MRS	50	45	45	9	9
	MRS-V	50	40	40	6	6
Oral mucosa	MRS	50	37	37	8	8
	MRS-V	50	39	39	5	5
Rumen	MRS	50	41	41	12	12
	MRS-V	50	38	38	9	9
Vaginal mucosa	MRS	50	29	29	6	6
	MRS-V	50	32	32	4	4
Silage	MRS	50	48	48	10	13
	MRS-V	50	45	45	13	10
Total	-	500	394	394	82	82

Table 2. Origin of isolation and isolate media used for selected strains of LAB.

LAB	**Culture media**	**Origin**
Lactobacillus casei MSI1	MRS	Silage
Lactobacillus casei MSI5	MRS	Silage
Lactobacillus casei MRUV1	MRS-V	Cow´s rumen
Lactobacillus casei MRUV6	MRS-V	Cow´s rumen
Lactobacillus acidophilus MVA3	MRS	Cow´s vaginal mucosa
Lactobacillus nagelli MSIV4	MRS-V	Silage
Lactobacillus harbinensis MSI3	MRS	Silage
Lactobacillus harbinensis MSIV2	MRS-V	Silage
Lactobacillus fermentum SIVGL1	MRS-V	Silage
Lactobacillus plantarum MLE5	MRS	Cow´s milk
Lactobacillus plantarum MSI2	MRS	Silage
Pediococcus pentosaceus MLEV8	MRS-V	Cow´s milk
Pediococcus acidilactici MSI7	MRS	Silage
Corynebacterium vitaeruminis MRU4	MRS	Cow´s rumen
Weissella paramesenteroides MRUV3	MRS-V	Cow´s rumen
Weissella paramesenteroides MSAV5	MRS-V	Cow´s oral mucosa

* MRS-V: MRS added with vancomycin 10 mg/L.

Table 3. Enzymatic profile of the studied 16 LAB strains determined by APIZYM test.

LAB	Alkaline phosphatase	Esterase	Esterase lipase	Lipase	Leucine arilamidase	Valine arilamidase	Cistine arilamidase	Trypsin	α- chymotrypsin	Acid phosphatase	Naphthol phosfohydrolase	α- galactosidase	β- galactosidase	β- glucuronidase	α- glucosidase	β- glucosidase	N-acetyl-β-glucosaminidase	α- manosidase	α- fucosidase
Lactobacillus casei MSI1	-	+	+	-	+	+	-	-	-	+	+	+	+	-	+	+	-	-	-
Lactobacillus casei MSI5	+	+	+	-	+	+	+	-	+	+	+	-	+	-	+	+	+	-	-
Lactobacillus casei MRUV1	-	-	-	+	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-
Lactobacillus casei MRUV6	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-
Lactobacillus acidophilus MVA3	-	+	+	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-	-
Lactobacillus nagelli MSIV4	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-
Lactobacillus harbinensis MSI3	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	-
Lactobacillus harbinensis MSIV2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Lactobacillus fermentum SIVGL1	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-
Lactobacillus plantarum MLE5	+	+	+	-	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-
Lactobacillus plantarum MSI2	-	+	+	-	+	+	+	-	-	+	+	-	+	-	+	+	+	-	-
Pediococcus pentosaceus MLEV8	-	+	+	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-
Pediococcus acidilactici MSI7	-	-	-	-	+	+	+	+	-	+	+	-	+	-	-	+	+	-	-
Corynebacterium vitaeruminis MRU4	-	+	+	-	+	-	-	-	+	+	+	+	-	-	-	+	-	-	-
Weissella paramesenteroides MRUV3	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-	+	+	-	-
Weissella paramesenteroides MSAV5	-	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	-	-	-

*** alkp: alkaline phosphatase; est: esterase; estl: esterase lipase; lip: lipase; leua: leucine arilamidase; vala: valine arilamidase; cisa: cistine arilamidase; try: trypsin; α-chy: α-chymotrypsin; acip: acid phosphatase; napp: naphthol phosfohydrolase; α-gal: α-galactosidase; β-gal: β-galactosidase; β-glu: β-glucuronidase; α-glu: α-glucosidase; β-glu: β-glucosidase; N-β-g: N-acetyl-β-glucosaminidase; α-ma: α-manosidase; α-fu: α-